Tecan Symposium 2008

Lonza

High-Throughput Cell Engineering as a Tool in Research and Drug Development

Herbert Müller-Hartmann
Director R&D
amaxa AG, a Lonza company

Lonza Strategy Focus on Cell Discovery

Cells

Normal human cells
Adult and embryonic stem cells
Disease cells
« Cloned cells » SFM media ready

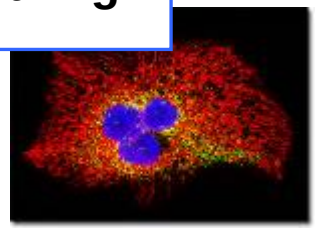
Cell based assays

Assays in cell

- Cell based HTS/HCS assay
- In Vitro ADMET assays
- Cell Health assays
- Cell function assays

Cell handling

Ease of use



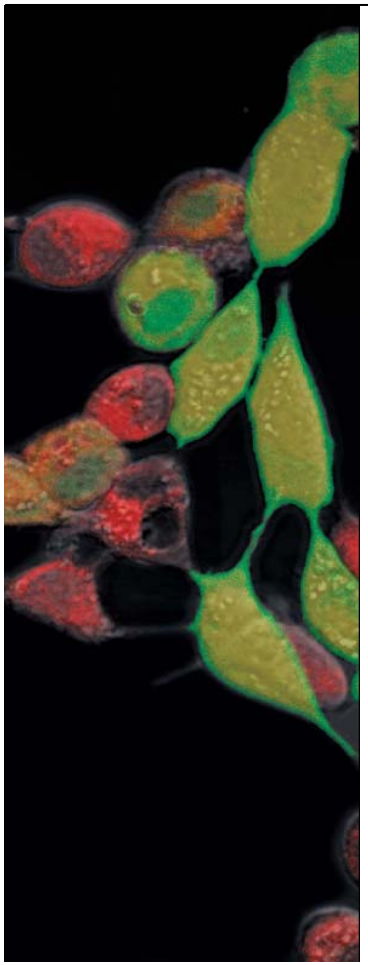
- Dedicated culture media
- Serum Free Media
- Cell culture matrices
- Innovative cell culture support

Cell analysis

Enabling Technology

- Transfection
- Silencing RNAs
- Gene expression arrays
- Cellular imaging and analysis

Cell Engineering in High Throughput



Agenda

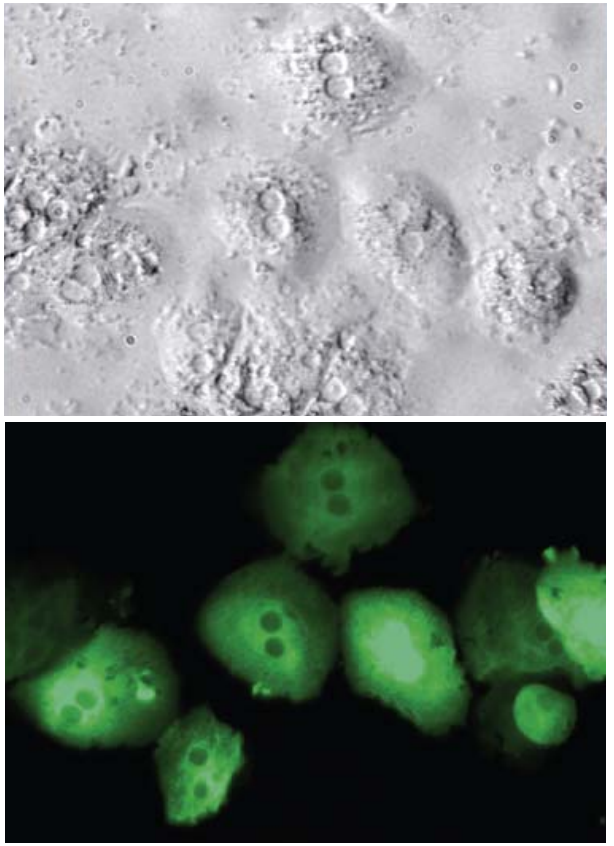
- **High-Throughput Applications Based on Cell Engineering**
- Functional siRNA Screening
  - Kinome/Cell Cycle siRNA Screening in Human Umbilical Vein Endothelial Cells
  - Functional Fas-apoptosis Screen in Jurkat T Lymphocytes
- Cell Engineering with Non-Nucleic Acid Molecules

Lonza

High-Throughput Transfection and Engineering of Cells by Chemical, Viral and Physical Methods

Leading to more efficient

- Target research
- Lead identification and valuation
- Protein production
- Optimization of vectors and methods



Primary human hepatocytes

Müller-Hartmann *et al.*, Expert Opin. Drug Discov. 2:1453-1465, 2007

High-Throughput Cell Engineering

Developed for HT

Methods

- Electrofusion
- Physical delivery
  - Electroporation
  - Magnetofection
  - Sonoporation
  - Laser irradiation
  - Microinjection
  - Ballistic delivery
  - …
- Chemical delivery
- Lipofection
  - Cationic polymers
  - Calcium phosphate transfection
- Viral transduction
  - Lentivirus
  - Adenovirus
  - Adeno-associated virus

Müller-Hartmann *et al.*, Expert Opin. Drug Discov. 2:1453-1465, 2007

Lonza

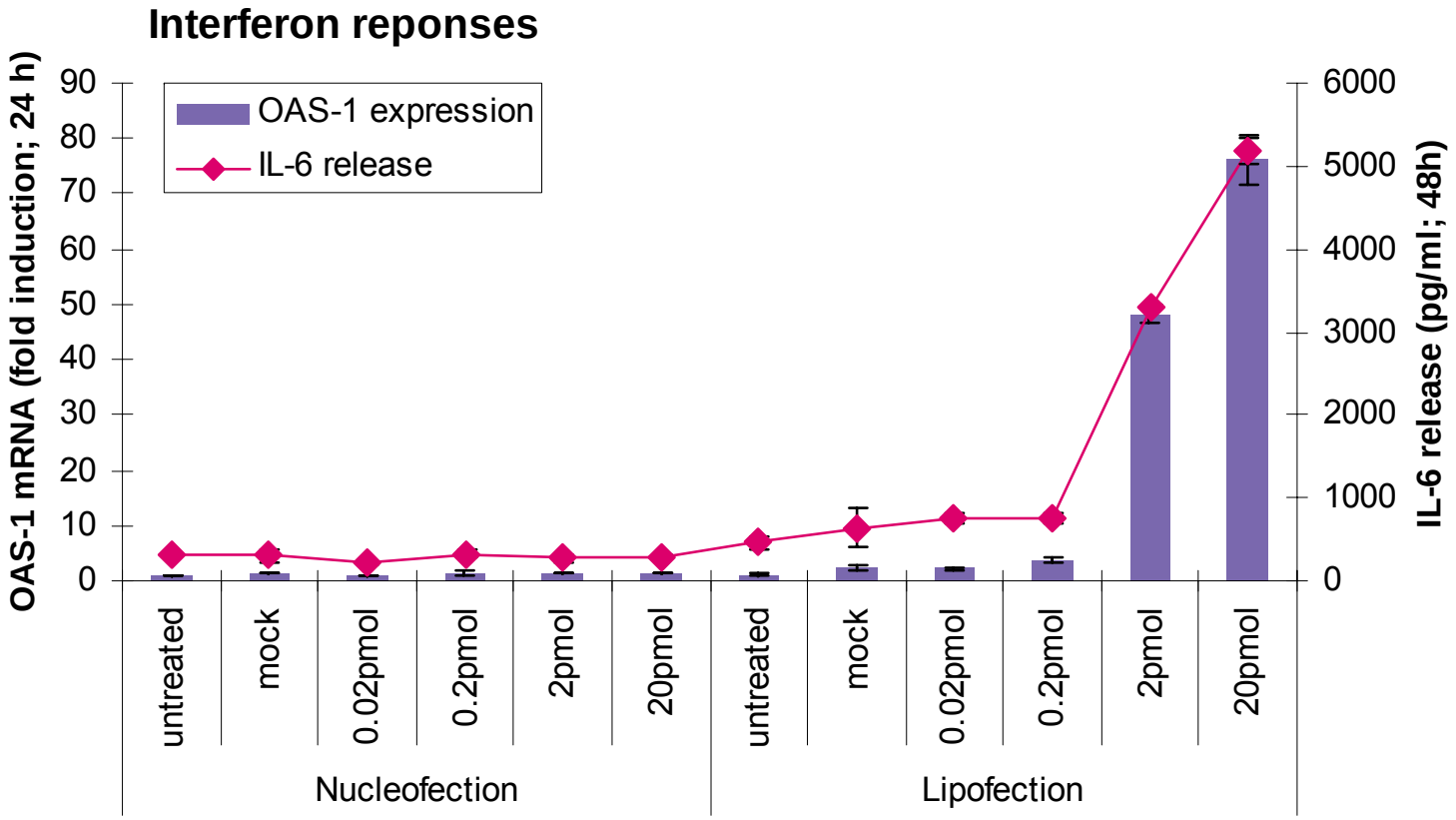
High-Throughput Cell Engineering

- Increasing utilization of cell-based-assays, supplementing and even replacing biochemical assays

- Recent trends in cell engineering
  - Biologically relevant cells, in particular primary cells
  - “Biologically inert” manipulation methods (e.g. “endosome-free” delivery)
  - “Cells as reagents”

Interferon Responses: Related to the Method of Delivery

HeLaS3 cells transfected with a 29-mer siRNA targeting DBI

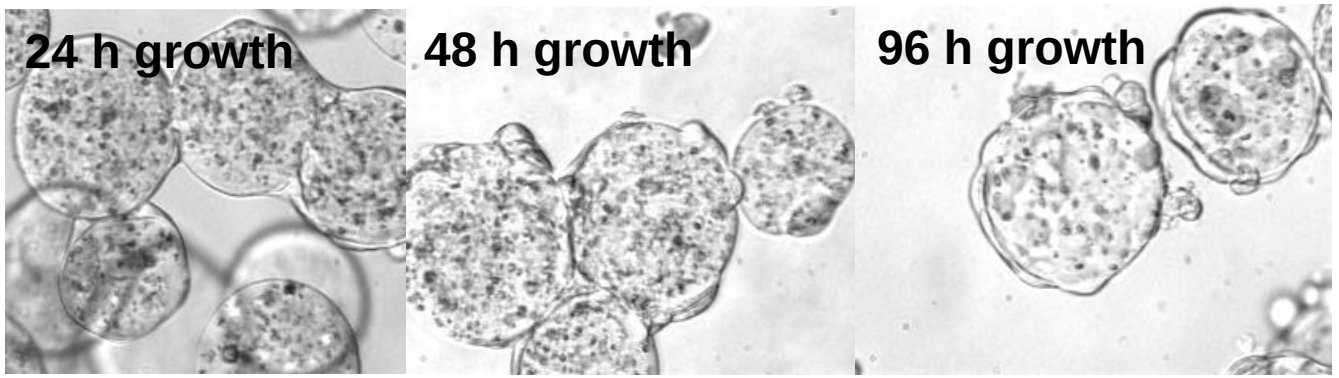


Data generated in collaboration with DHARMACON

Lonza

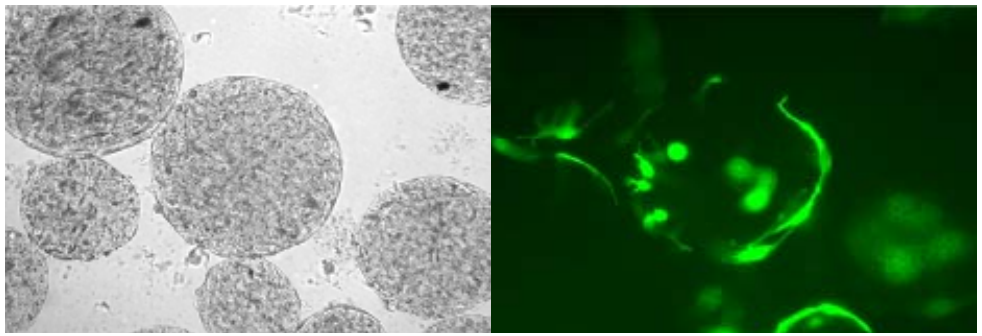
“Cells as reagents”

Cells show good growth and morphology on GEM™ beads



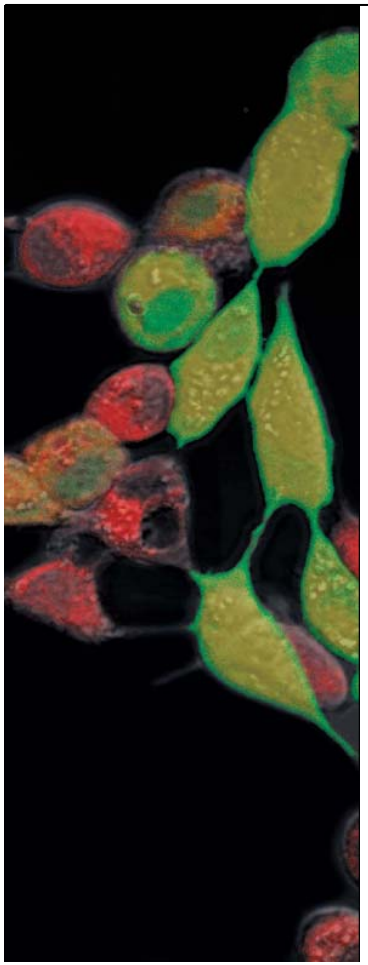
CHO-K1

Proof-of-principle transfection of cells growing on alginate beads



AoSMC

Cell Engineering in High Throughput



Agenda

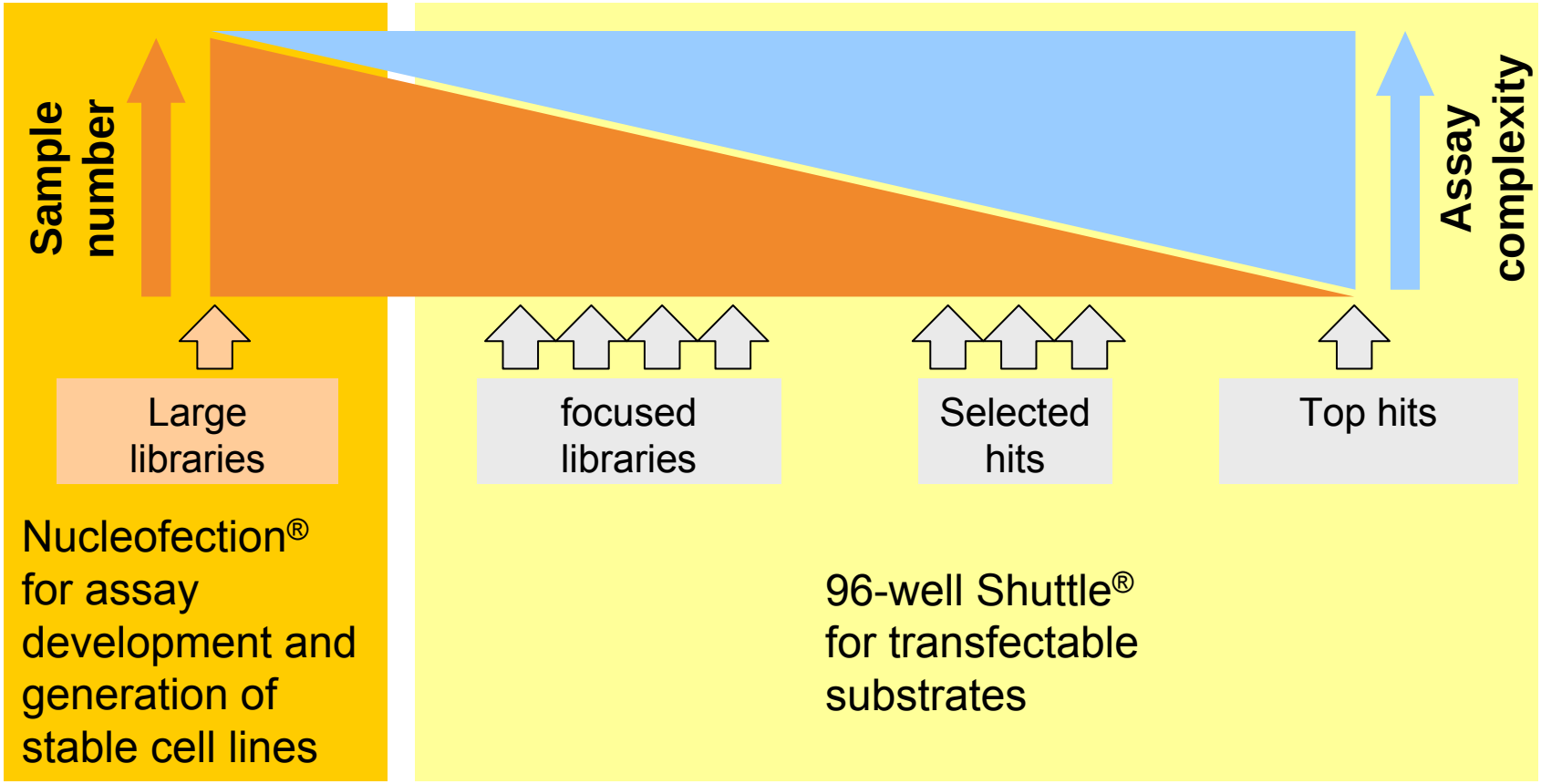
- High-Throughput Applications Based on Cell Engineering
- **Functional siRNA Screening**
  - Kinome/Cell Cycle siRNA Screening in Human Umbilical Vein Endothelial Cells
  - Functional Fas-apoptosis Screen in Jurkat T Lymphocytes
- Cell Engineering with Non-Nucleic Acid Molecules

RNAi Screening Strategies

- Loss/Gain of function screens
  - KD of target by RNAi induces phenotype (e.g. Proliferation / cell death)
- Modifier / sensitizer screens
  - KD of target by RNAi induces phenotype only in combination with cell treatment (e.g. Pathway induction, cell stress)
- Synthetic lethality screens
  - KD of target by RNAi induces phenotype only in certain genetic background (e.g. Comparison of isogenic cell model with wt-Ras vs. Ras-mutated)
- Pathway-specific screens using reporter gene
  - KD of target modulates signaling measured by pathway-specific reporter gene (stable reporter cell lines)

Cell-based Screening Approaches

Workflow & opportunities for Amaxa® Nucleofection®



Assay Types

- Viability
- Proliferation
- Apoptosis

- Calcium Assays
- ELISA
- Flash-Luminescence
- Reporter Gene assays

- High Content Analysis (HCA)
- Protein-Protein-interaction (e.g. FRET)
- Pathway specific assays

- Standard assays
- Commercially available
- Easy to optimize
- Easy to automate
- Standard instrumentation

- High optimization effort
- Special Instrumentation

Automation Solutions

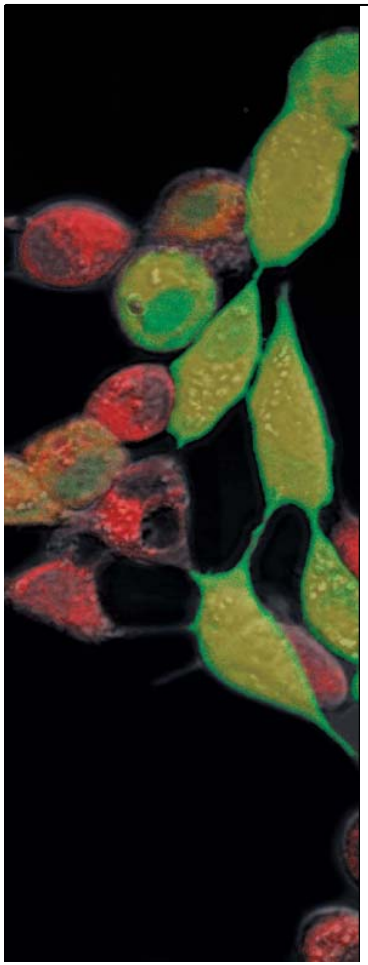
- Workflow considerations
- Depending on number of samples, cells have to be stored in Nucleofector® solution for hours
  - Available automation friendly OPs

Cell types	Conditions
Jurkat	modified protocol
HeLa/HeLa-S3	modified protocol
Neuro2A	modified protocol
HUVEC	Standard OP
human T-cells new OP	Standard OP
human T-cells old OP	Standard OP
stim. human T-cells	Standard OP

More to come …

If no automation OP available: check for stability in solution, contact Amaxa

Cell Engineering in High Throughput



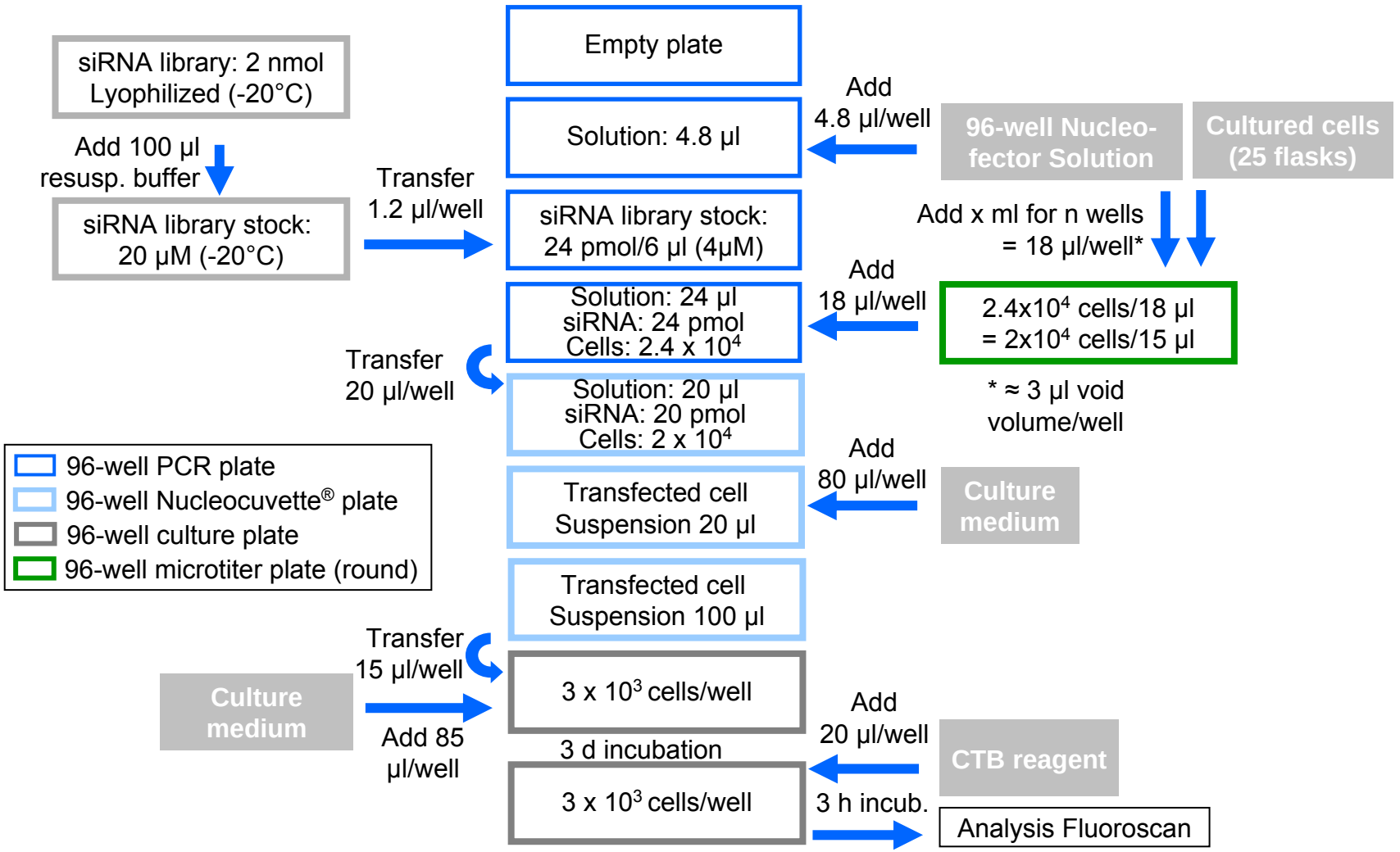
Agenda

- High-Throughput Applications Based on Cell Engineering
- Functional siRNA Screening
  - **Kinome/Cell Cycle siRNA Screening in Human Umbilical Vein Endothelial Cells**
  - Functional Fas-apoptosis Screen in Jurkat T Lymphocytes
- Cell Engineering with Non-Nucleic Acid Molecules

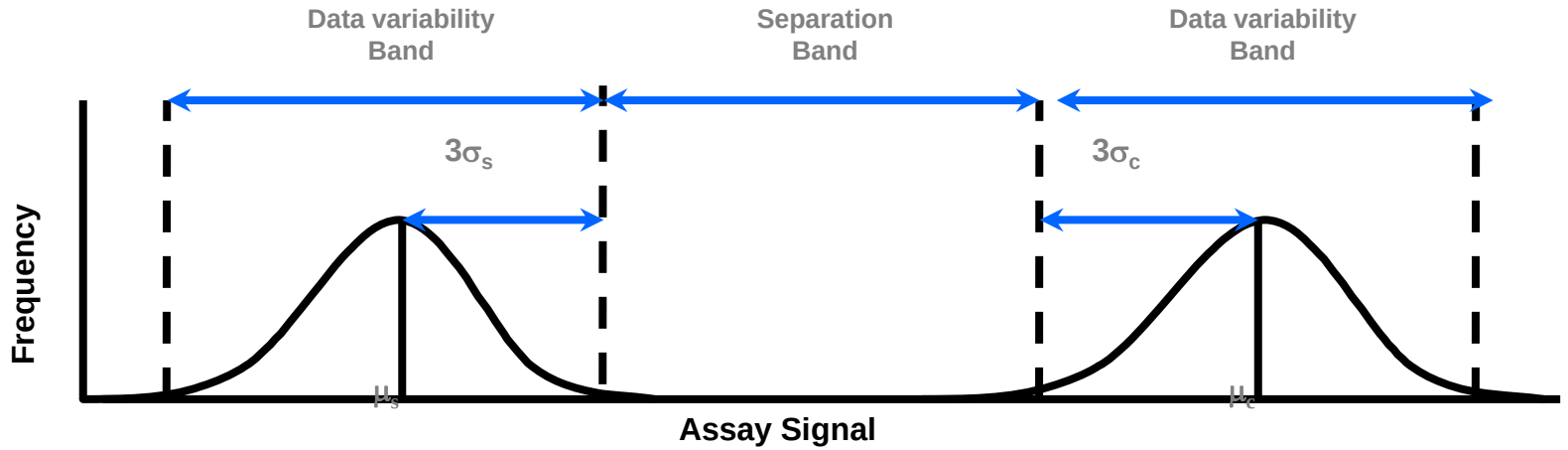
siRNA Screen in Primary HUVEC Cells

siRNA libraries	■ Human siARRAY® - Protein Kinases (Dharmacon): SMARTpool® siRNA targeting 779 genes ■ Human siARRAY® - Cell Cycle Regulation (Dharmacon): SMARTpool® siRNA targeting 111 genes
Controls	■ Untreated cells (in 96-well Solution only) ■ Negative control: siCONTROL® non-targeting siRNA #1 (Dharmacon) ■ Positive control: SMARTpool® targeting PLK-1 (Dharmacon) and CHEK1 (Dharmacon)
Analysis	■ Cell viability using CellTiter-Blue® assay (Promega) ■ 72 h post transfection ■ Data analysis: ■ Z' factor of controls (quality of experiment) ■ Robust Z-score (hit identification)

Screening Workflow



Robustness and Screening Window



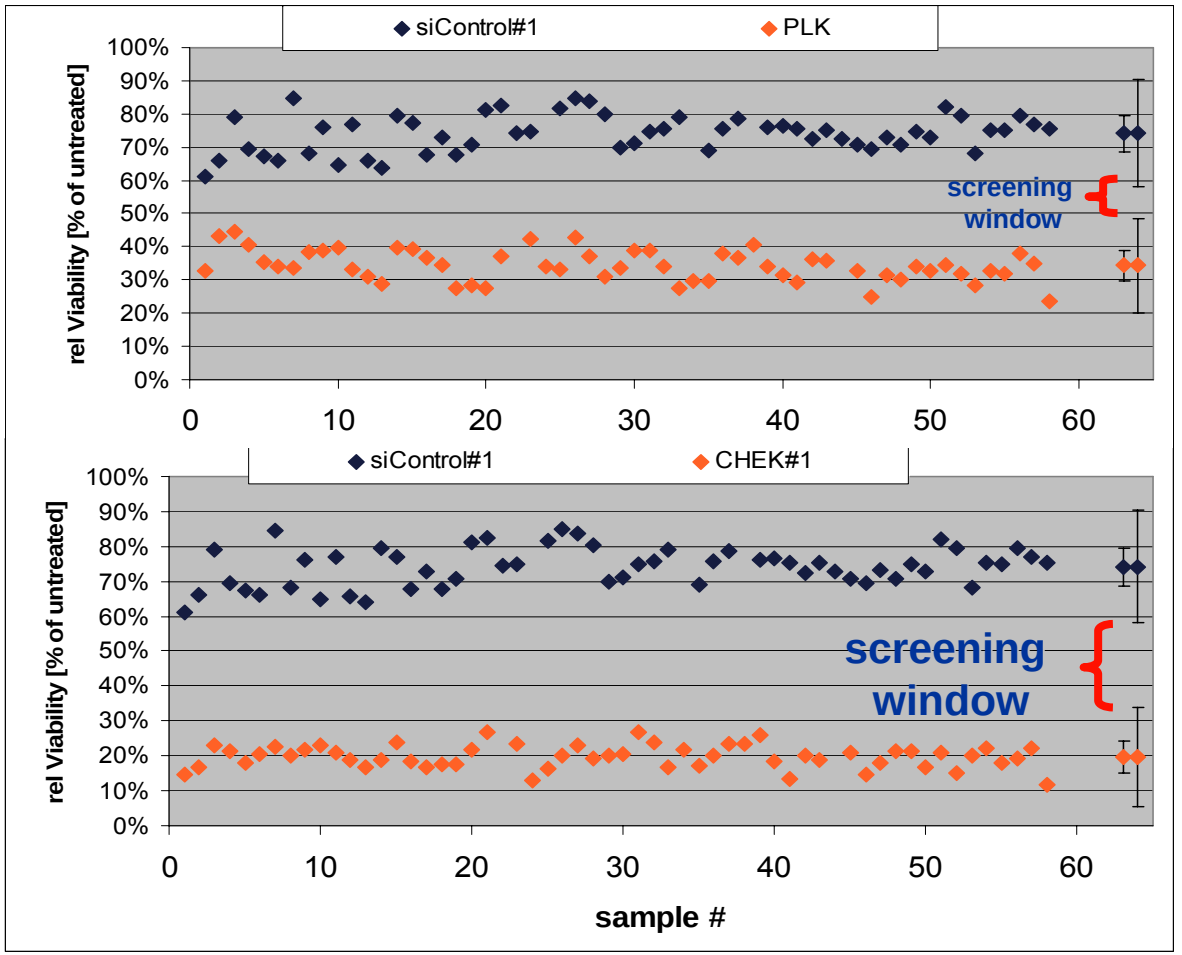
$$Z' = 1 - \frac{3SD_{positive} + 3SD_{negative}}{|\, mean_{positive} - mean_{negative} \,|}$$

siRNA Screen in Primary HUVEC Cells

Assay setup: Robustness – Screening Window

Z'-factor = 0.2

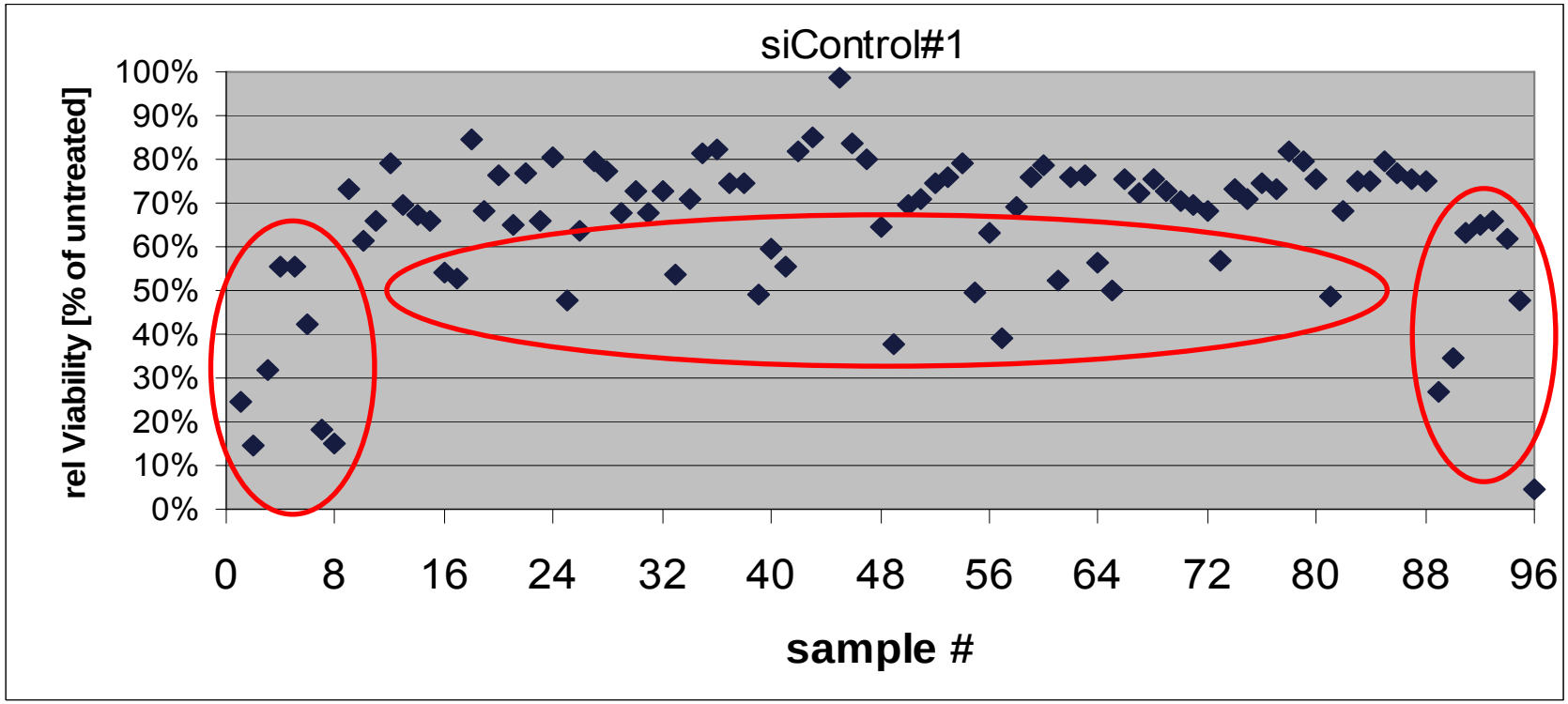
Z'-factor = 0.5



Data generated in collaboration with DHARMACON

siRNA Screen in Primary HUVEC Cells

Assay setup: Plate uniformity?

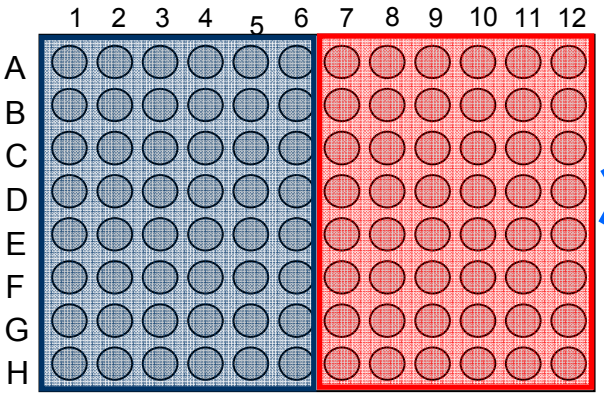


→ HUVEC cell readout shows position/edge effects!

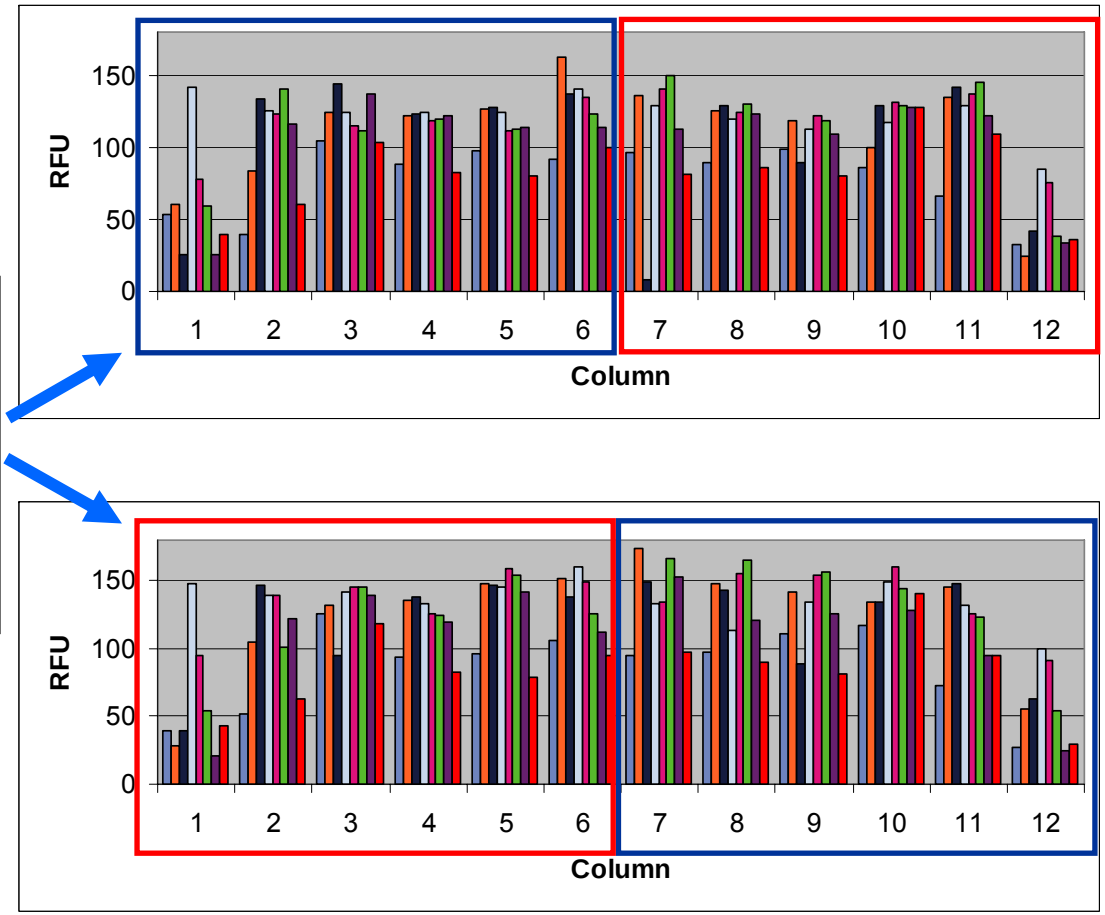
Data generated in collaboration with DHARMACON

Position / Edge Effects are Nucleofection® Independent

1 Nucleofection® sample was plated onto 2 culture plates in different orientations.

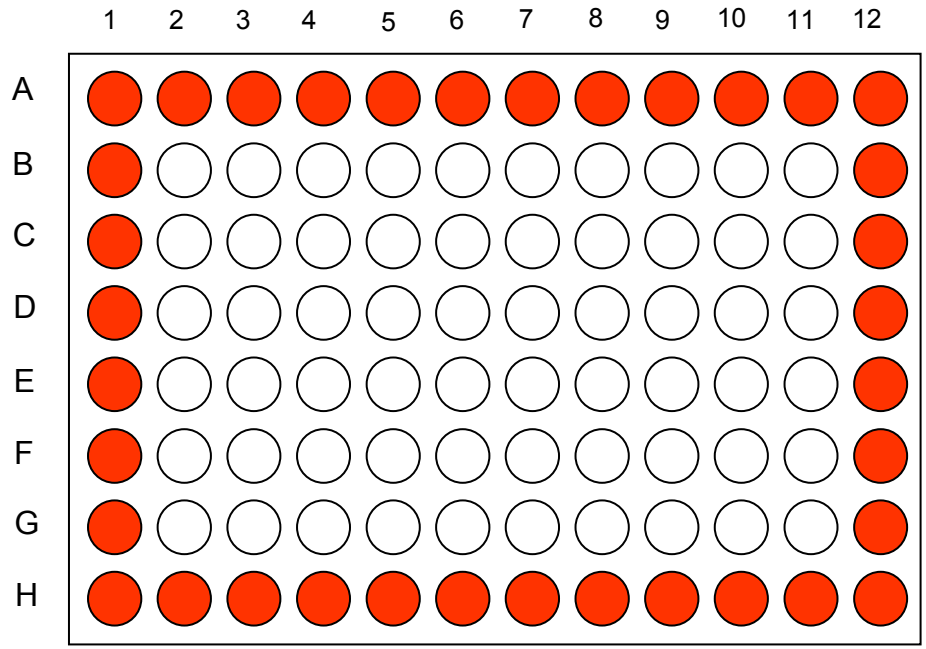


Nucleocuvette® Plate



Data generated in collaboration with DHARMACON

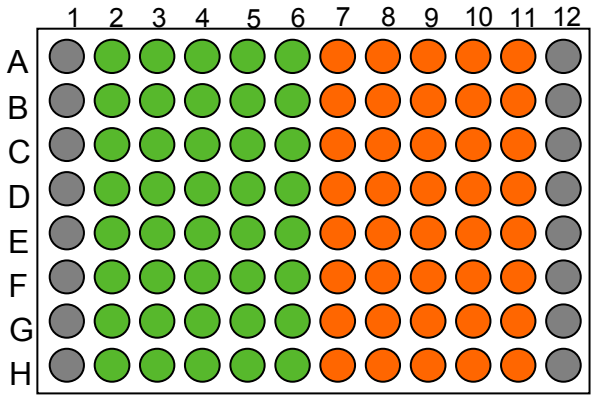
Performing the Screen While Omitting the Outer Wells Requires Reformatting of Library



Medium only

Reformatting Strategies for Nucleofection® II

Transfer from masterplate into Nucleocuvette® plate

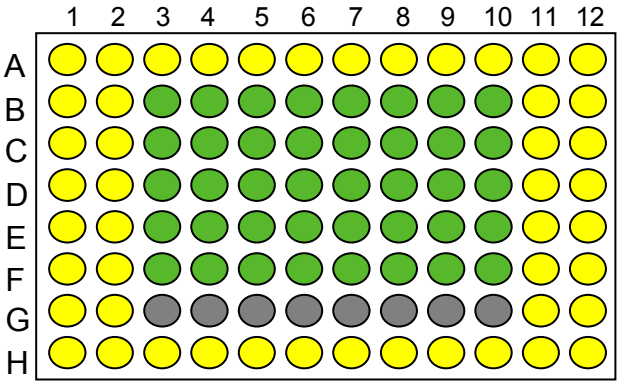


Reformat after Nucleofection® into 2 culture plates, medium in the outer wells is necessary!

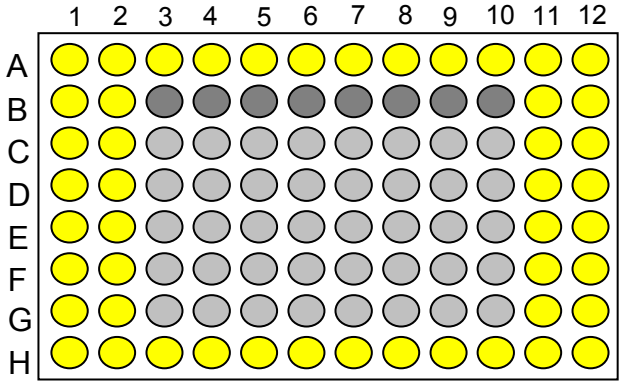
8 wells for controls

40 wells for targets

I

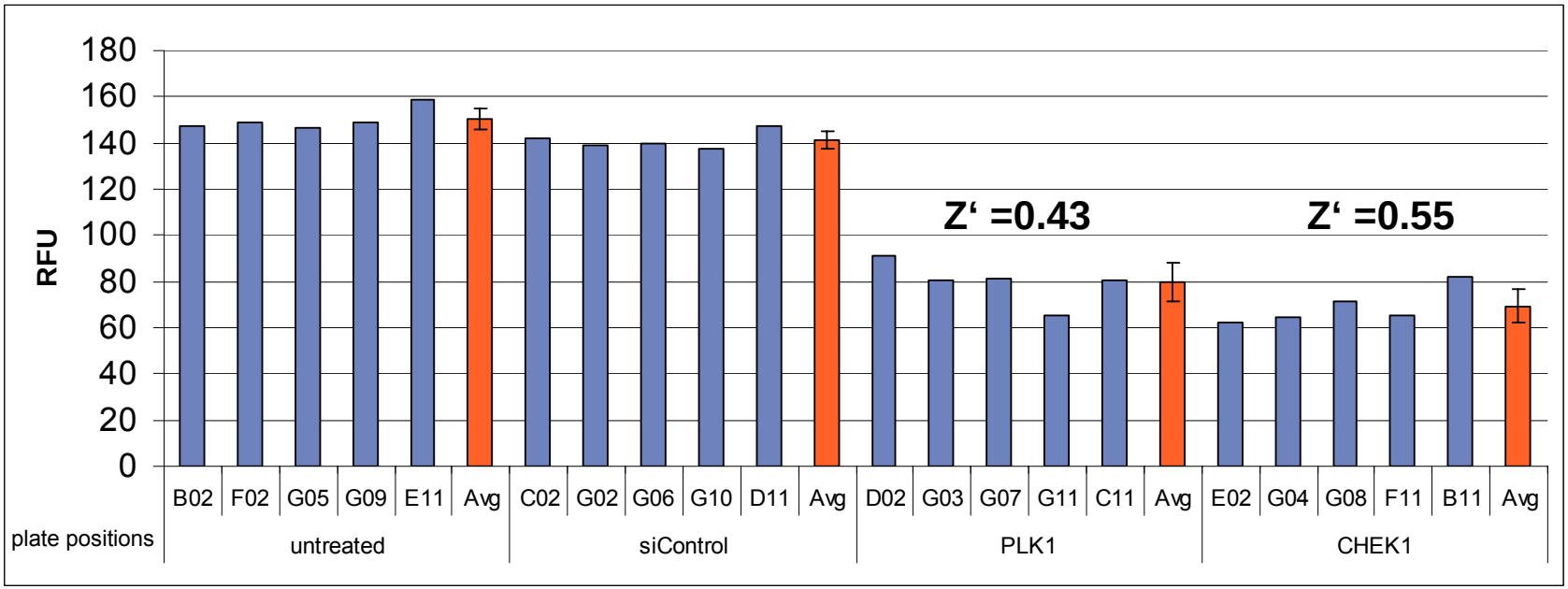


II



siRNA Screen in Primary HUVEC Cells

Primary Screen: Reproducibility of controls example plate

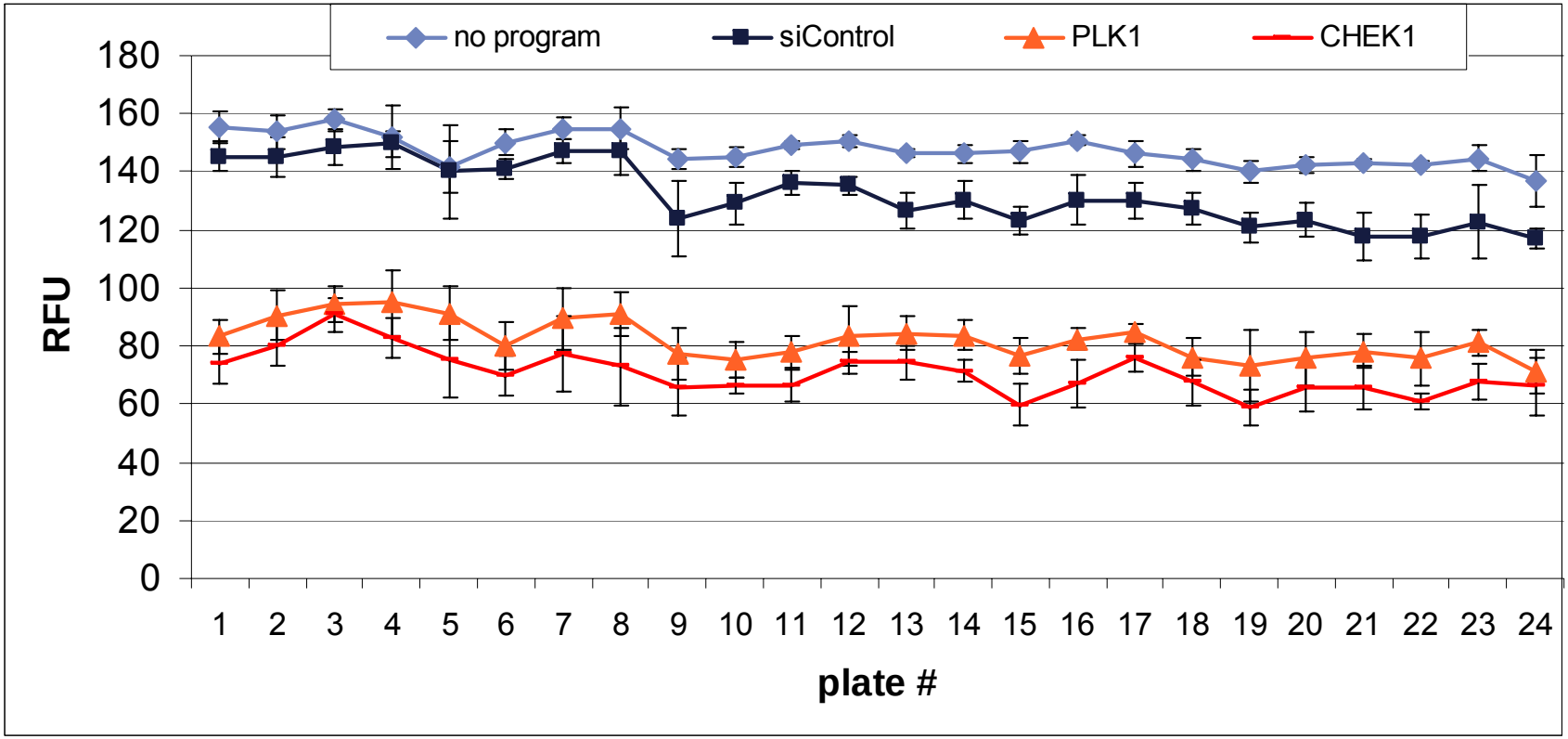


- **High reproducibility of controls** (untreated cells, siCONTROL®, PLK-1, CHEK1) **independent of plate position**

Data generated in collaboration with DHARMACON

siRNA Screen in Primary HUVEC Cells

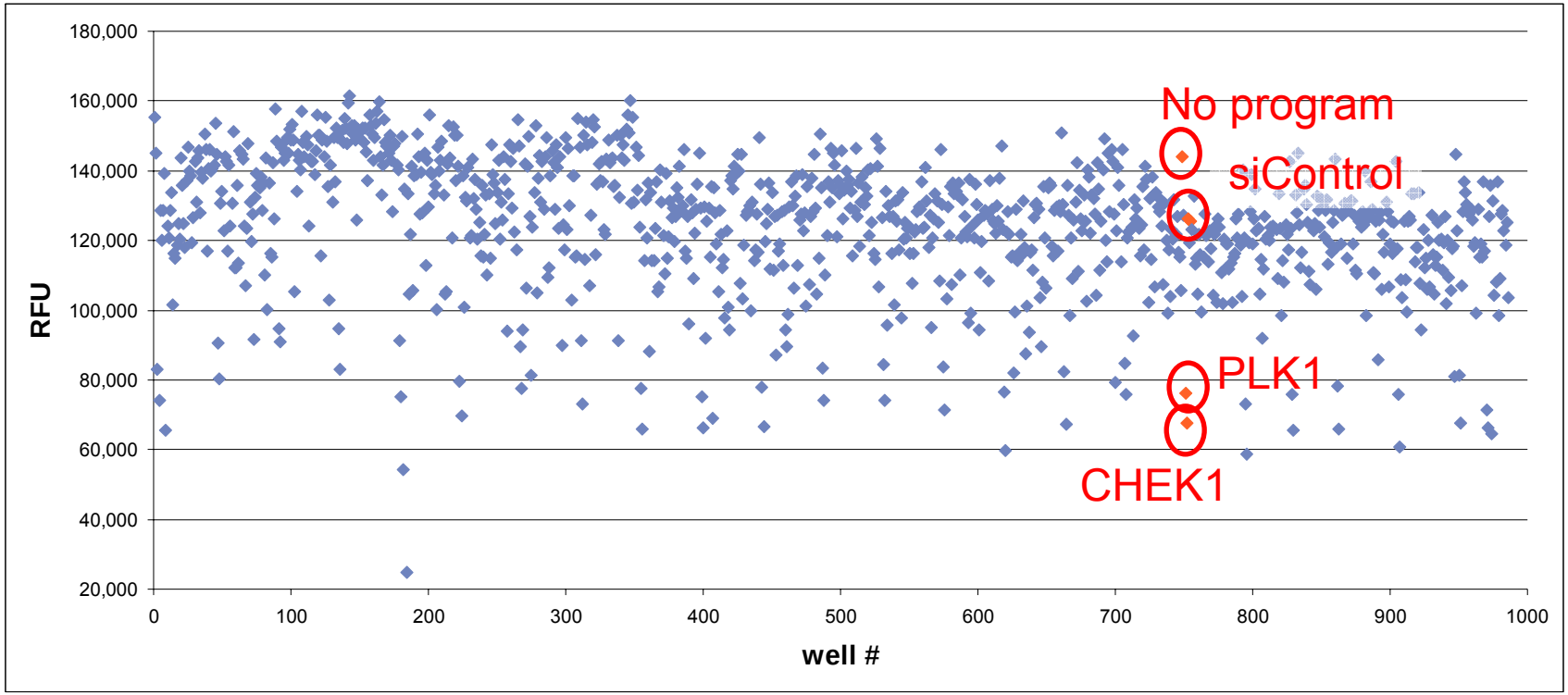
Primary Screen: Reproducibility of controls over all plates



Data generated in collaboration with DHARMACON

Data Processing

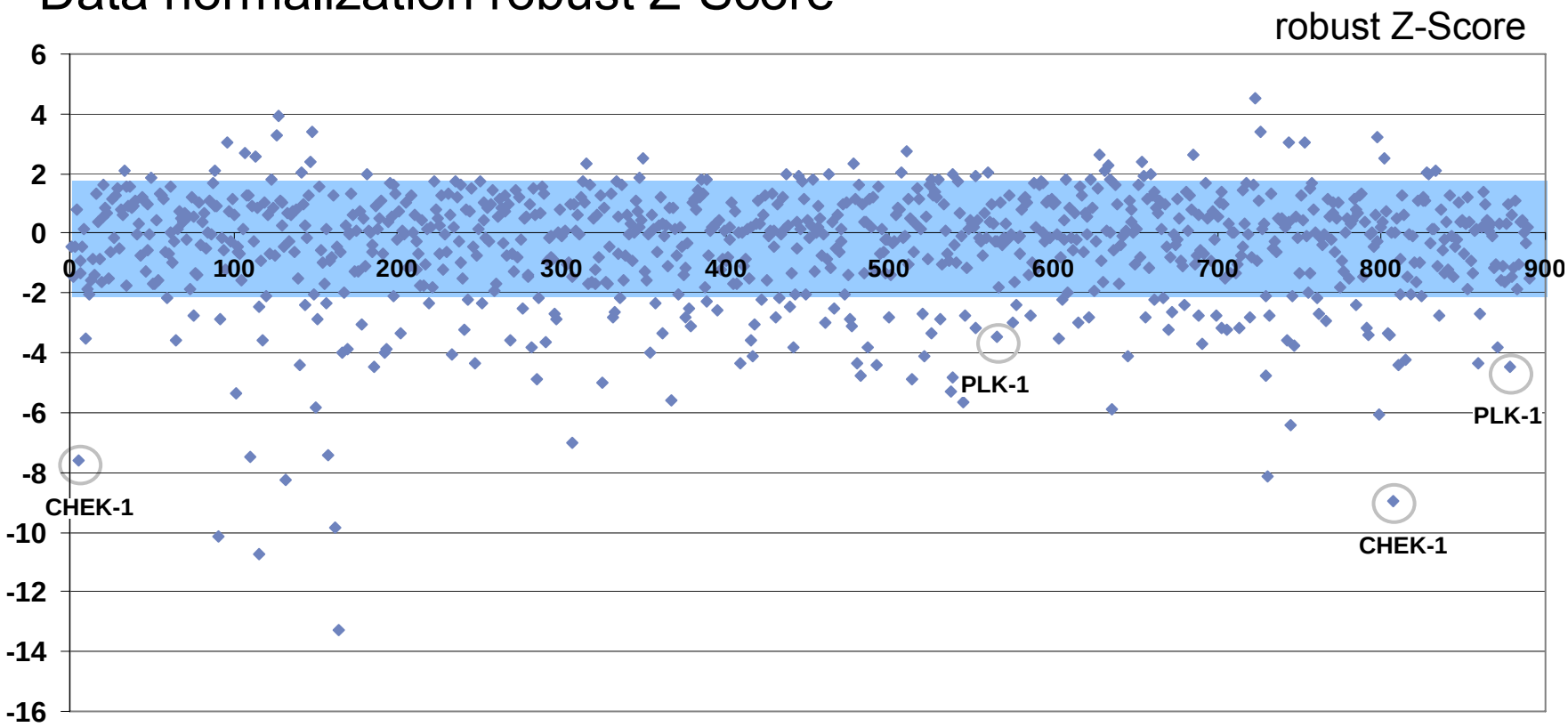
Raw Data



Data generated in collaboration with DHARMACON

Data Processing

Data normalization robust Z-Score

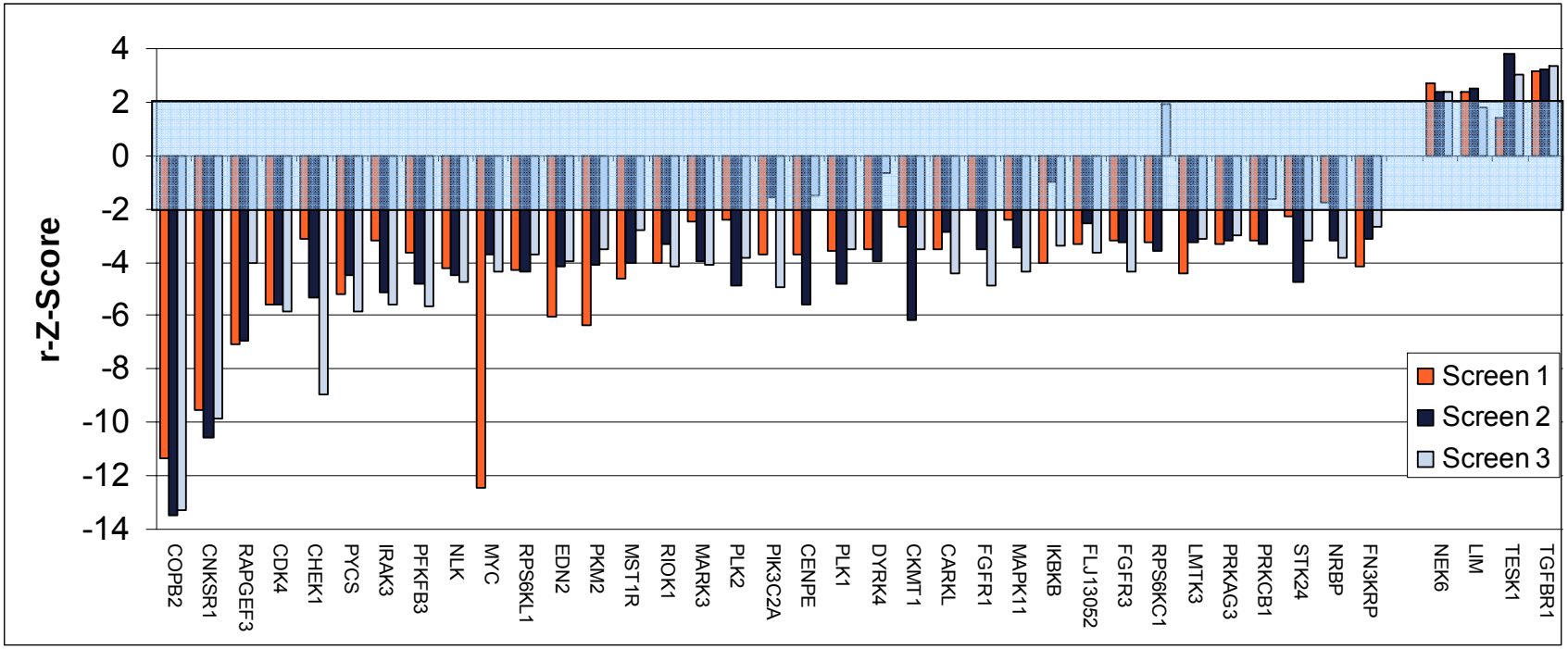


- In total > 40 primary hits showing reproducible effects >2x MAD of the plate mean

Data generated in collaboration with DHARMACON

siRNA Screen in Primary HUVEC Cells

Primary Screen: Comparison of three Screens



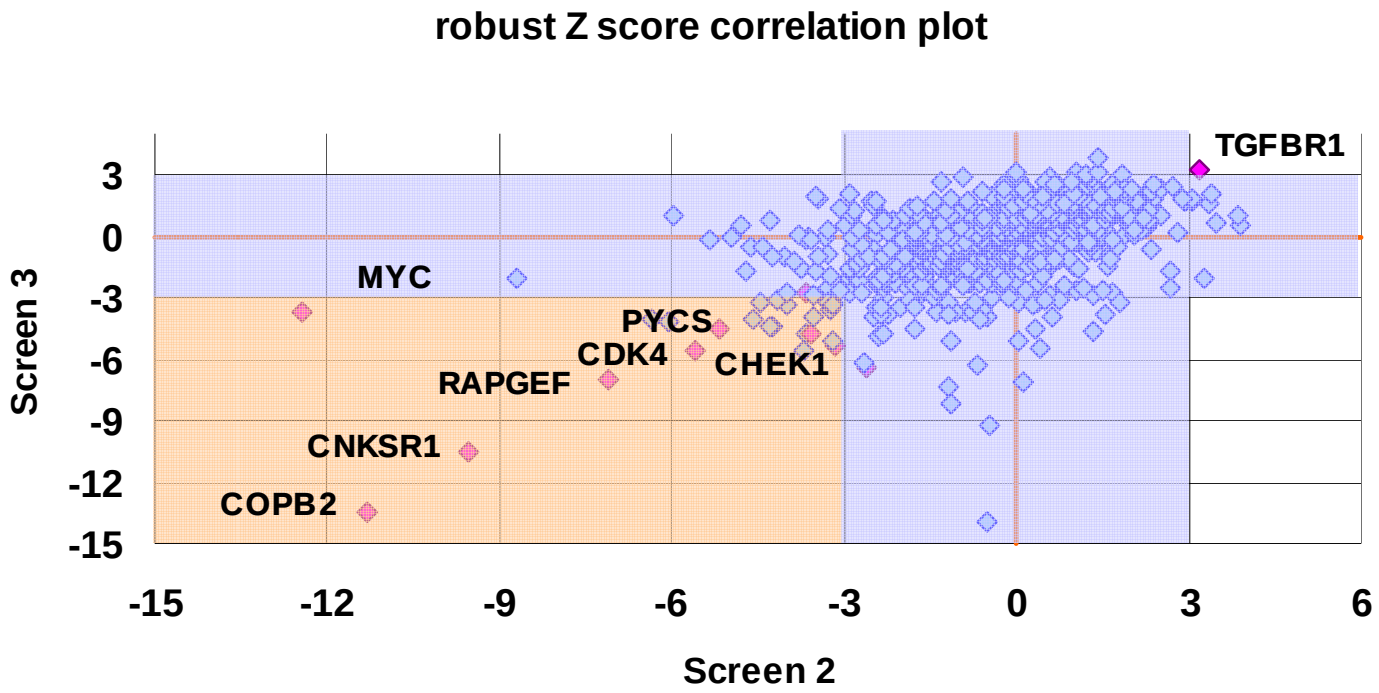
Hits sorted by rZ-Scores

→ Good correlation between individual screens

Data generated in collaboration with DHARMACON

siRNA Screen in Primary HUVEC Cells

Primary Screen: Correlation of two Screens



→ Good correlation between individual screens

Data generated in collaboration with DHARMACON

siRNA Screen in Primary HUVEC Cells
Validation Strategy

Lonza

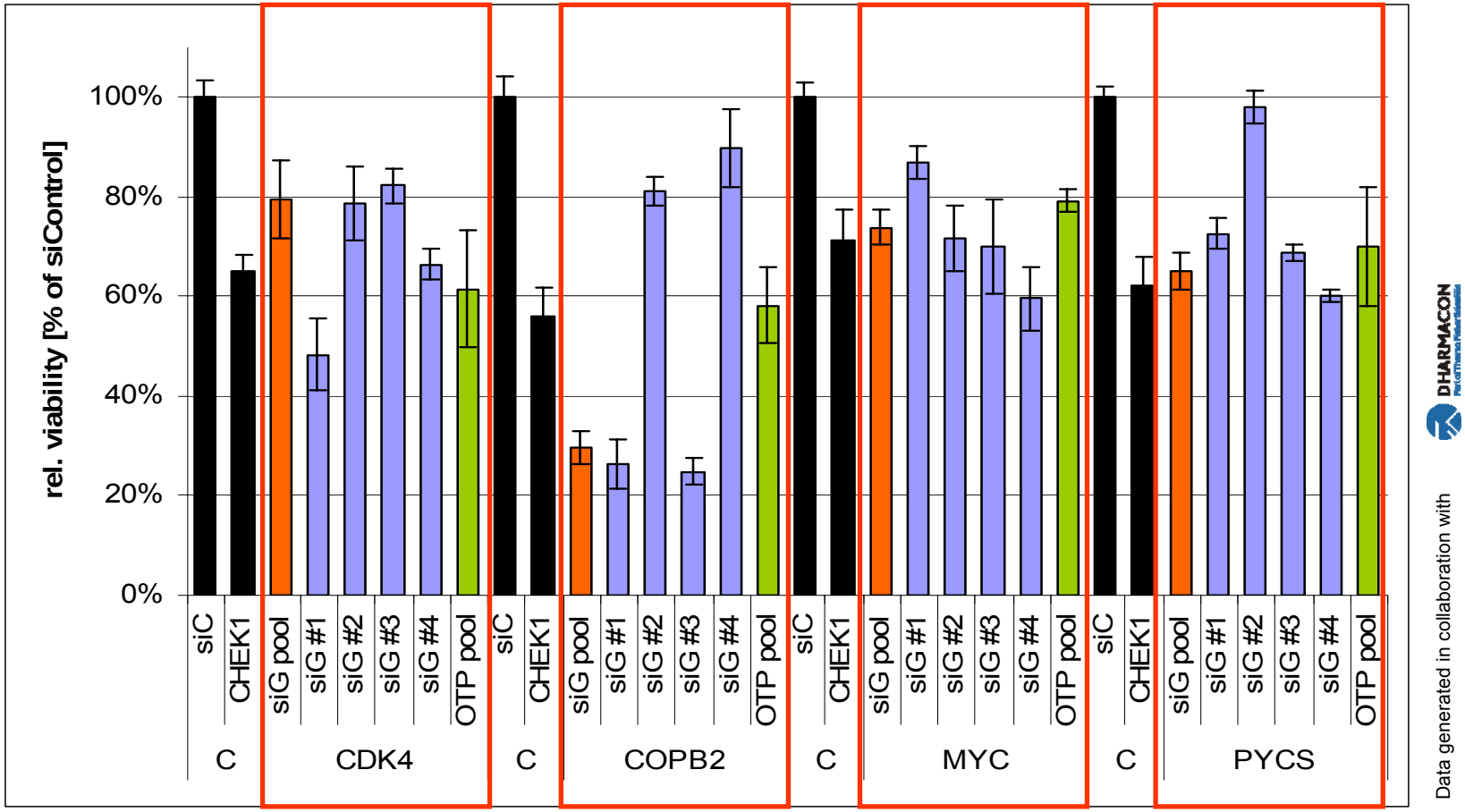
Confirmation

- Retest identified Hits with same substrate with higher n's

Redundancy

- De-convolution of siGENOME™ SMARTpool® (Dharmacon)
  - Test of 4 different single siRNAs
- OTP SMARTpool®
  - Different substrate, contains siRNA sequences different from the siGENOME™ SMARTpool®
- Dose Response

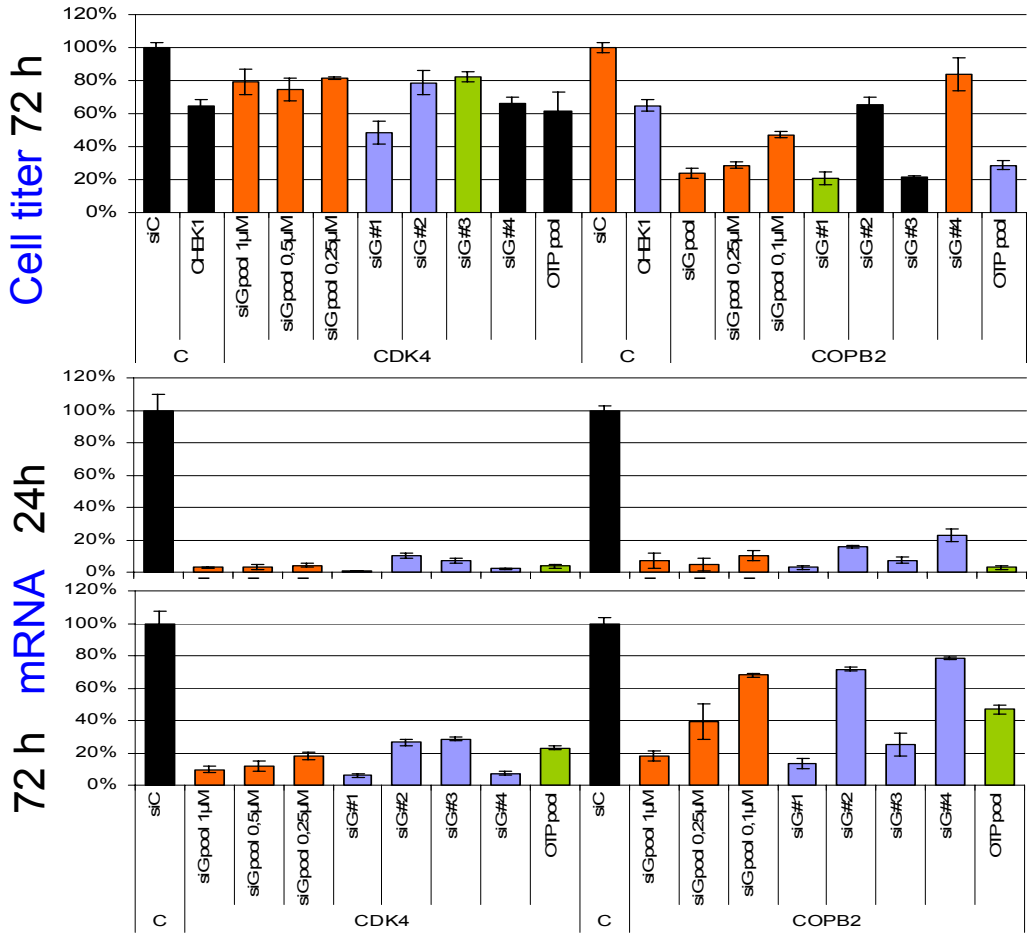
siRNA Screen in Primary HUVEC Cells
Validation of TOP Hits



Data generated in collaboration with DHARMACON

- 2-4 single siRNAs show same phenotype as the pool
- OTP pools also confirm phenotype (different sequences!)

Validation of TOP Hits
Deconvolution of pools, phenotype vs KD

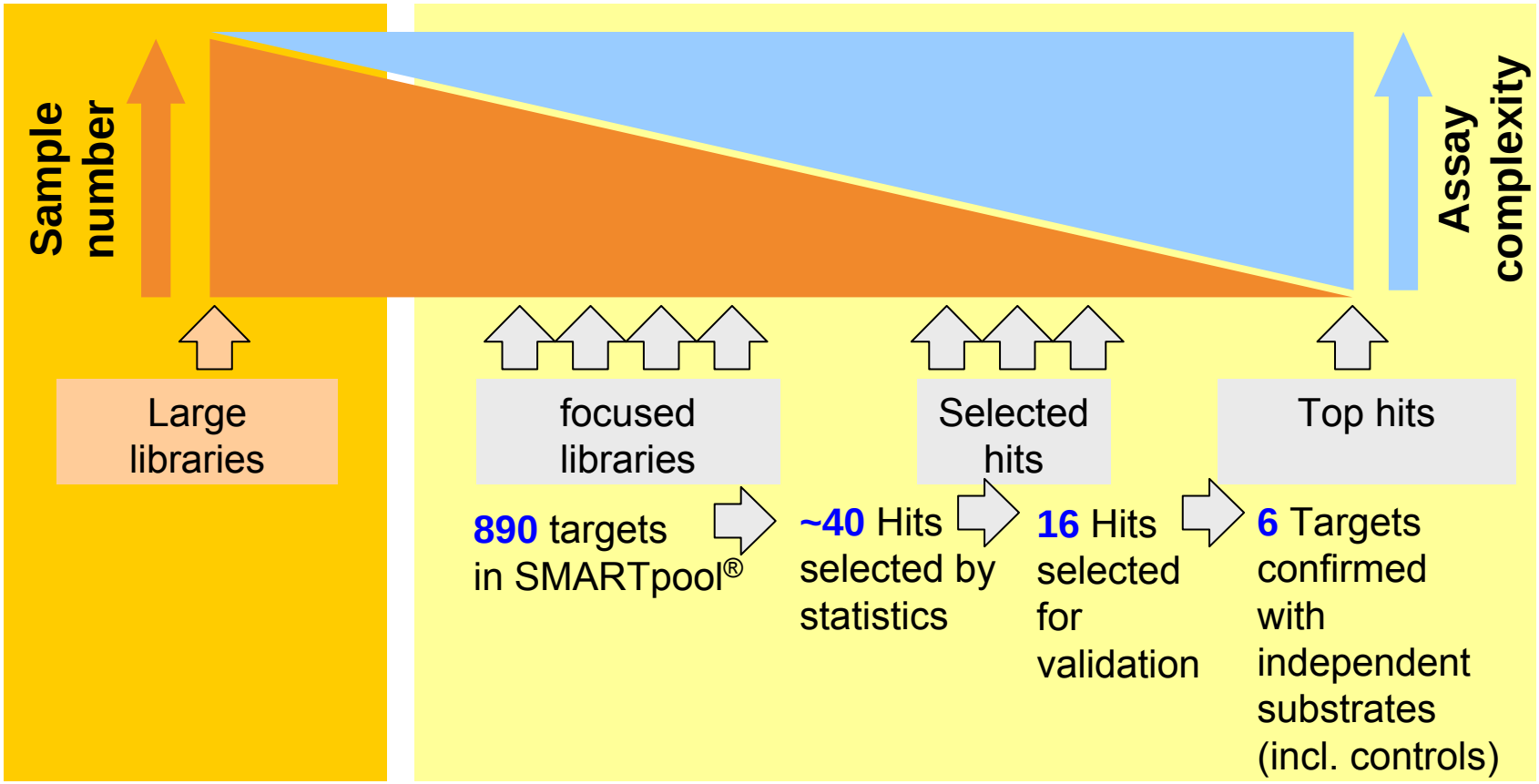


- Strong correlation of phenotype and knockdown CDK4
- OTP and 4 of 4 single siRNAs show phenotype, as well as, KD COPB2
- KD above 80% at 24h required for phenotype
- OTP and 2 of 4 single siRNAs show phenotype, as well as, KD

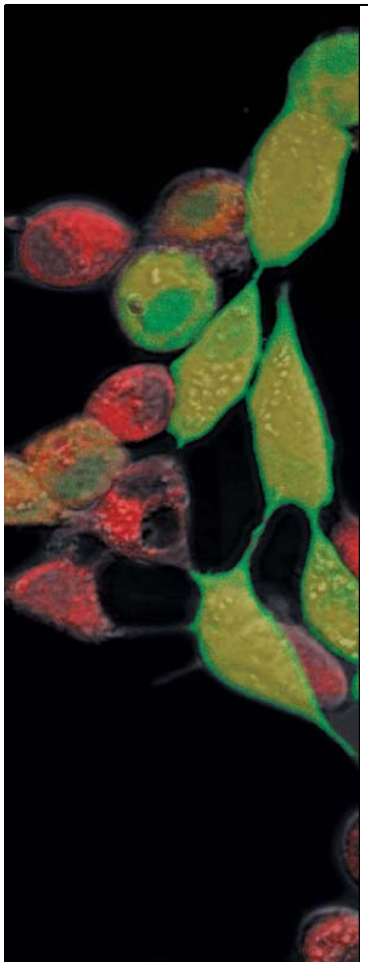
Data generated in collaboration with DHARMACON

siRNA Screen in Primary HUVEC Cells

Summary



Cell Engineering in High Throughput



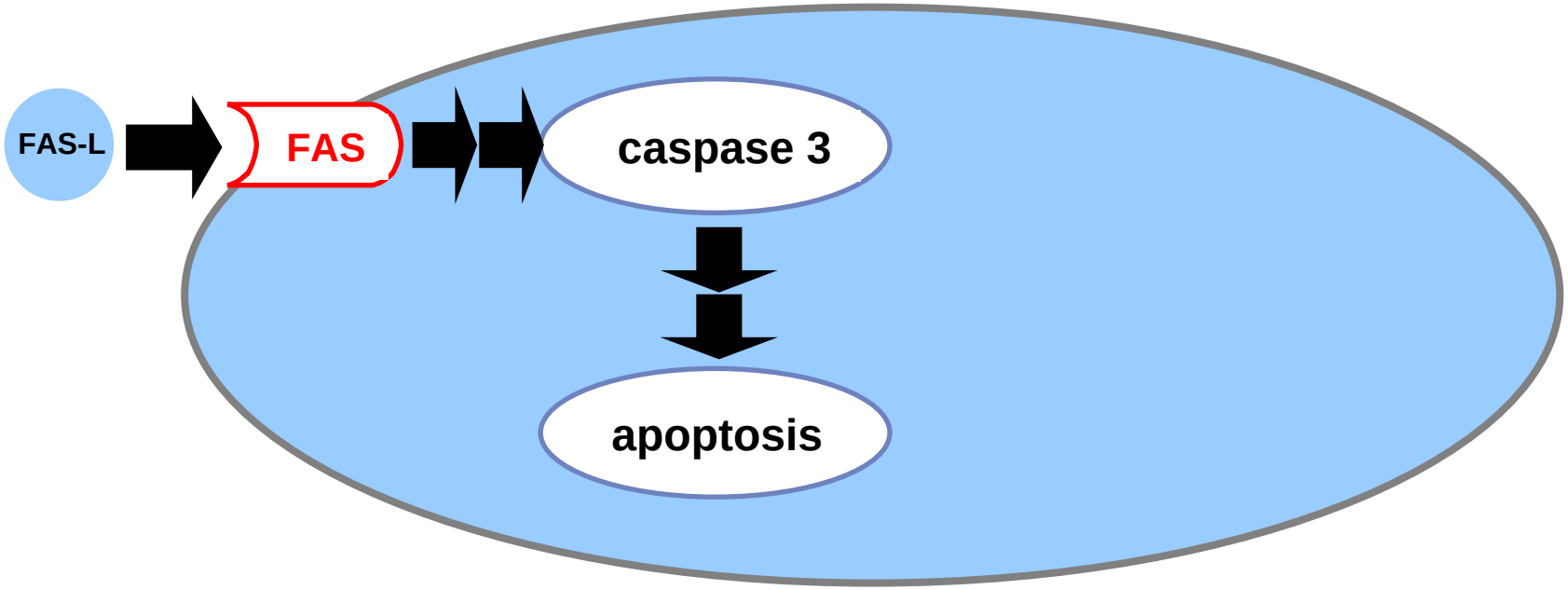
Agenda

- High-Throughput Applications Based on Cell Engineering
- Functional siRNA Screening
  - Kinome/Cell Cycle siRNA Screening in Human Umbilical Vein Endothelial Cells
  - **Functional Fas-apoptosis Screen in Jurkat T Lymphocytes**
- Cell Engineering with Non-Nucleic Acid Molecules

siRNA Screen in Jurkat T Lymphocytes

siRNA libraries	■ Human siARRAY® ON-TARGETplus™ Apoptosis (Dharmacon): SMARTpool® siRNA targeting 558 genes
Controls	■ Untreated cells (in 96-well Solution only) ■ Negative control: siCONTROL® non-targeting siRNA #1 (Dharmacon) ■ Positive controls: SMARTpool® ON-TARGETplus™ targeting FAS or Casp 3 (both Dharmacon)
Analysis	■ Induction of apoptosis with Fas-ligand 48h post transfection ■ Cell viability using CellTiter-Glo® assay (Promega) ■ Casp 3/7 using Apo-One® assay (Promega) ■ Data analysis: ■ Z' factor of controls (quality of experiment) ■ robust Z-score (hit identification)

FAS-Mediated Apoptosis

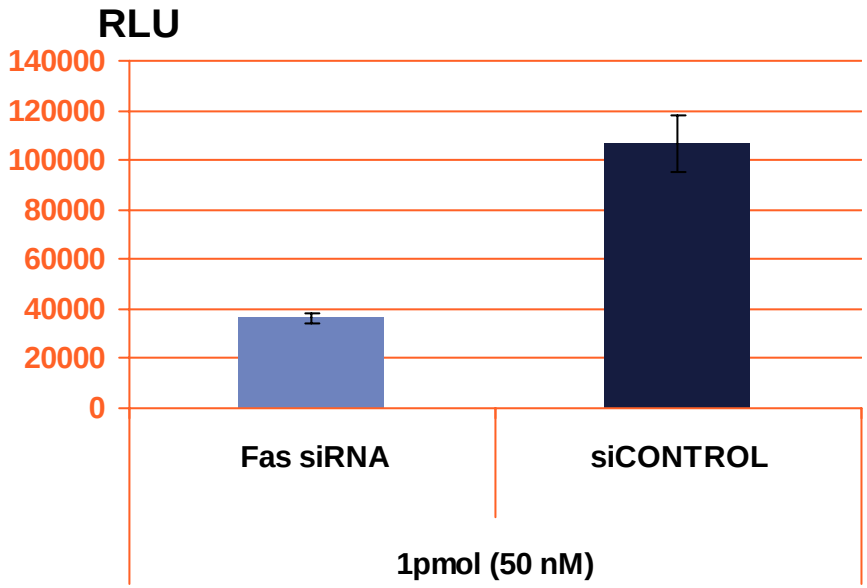


- Contributes to peripheral depletion of lymphoid cells
- Maintenance of self-tolerance
- Down-regulation of the immune response and homeostasis of the immune system

Phenotypic Knockdown of FAS-Mediated Apoptosis

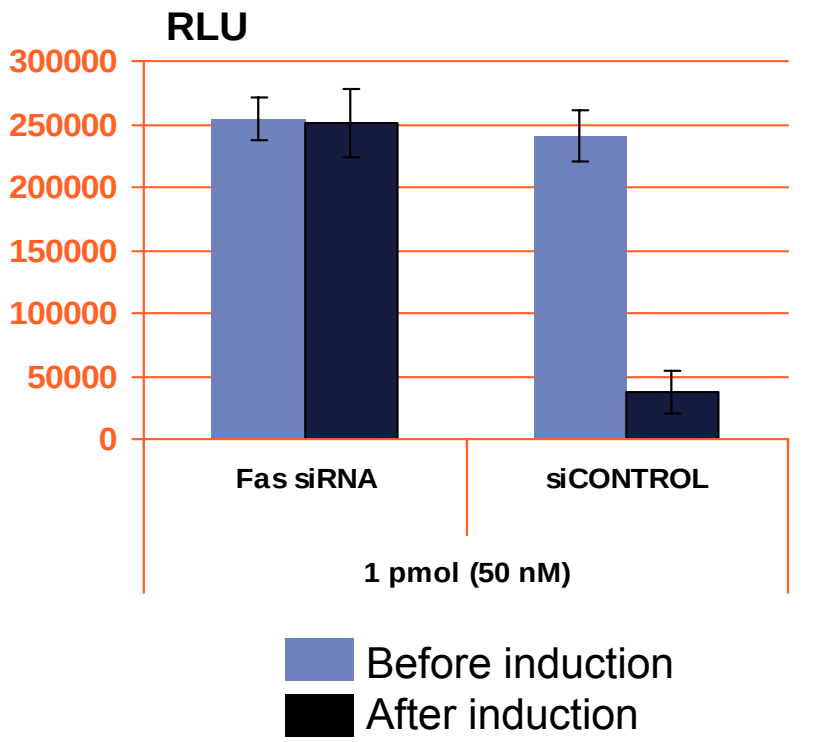
Caspase Activity

(Caspase-Glo™ Assay, Promega corp.)



Cell Viability Assay

(CellTiter-Glo® Assay, Promega corp.)



Data generated in collaboration with DHARMACON

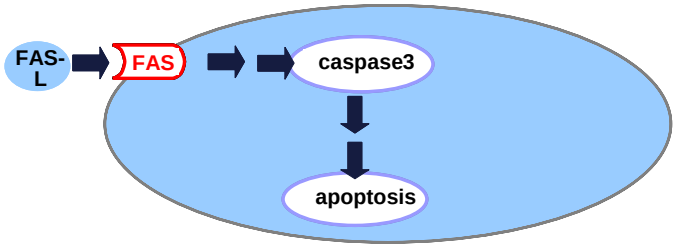
siRNA Screen in Jurkat T Lymphocytes

First Results

- First screen assays established only with FAS as positive control
  - Only FAS detected in screen
  - Responses from other pathway members too weak for consistent results
- Conditions were “tweaked” using different positive controls
  - FAS apoptosis screen repeated

siRNA Screen in Jurkat T Lymphocytes

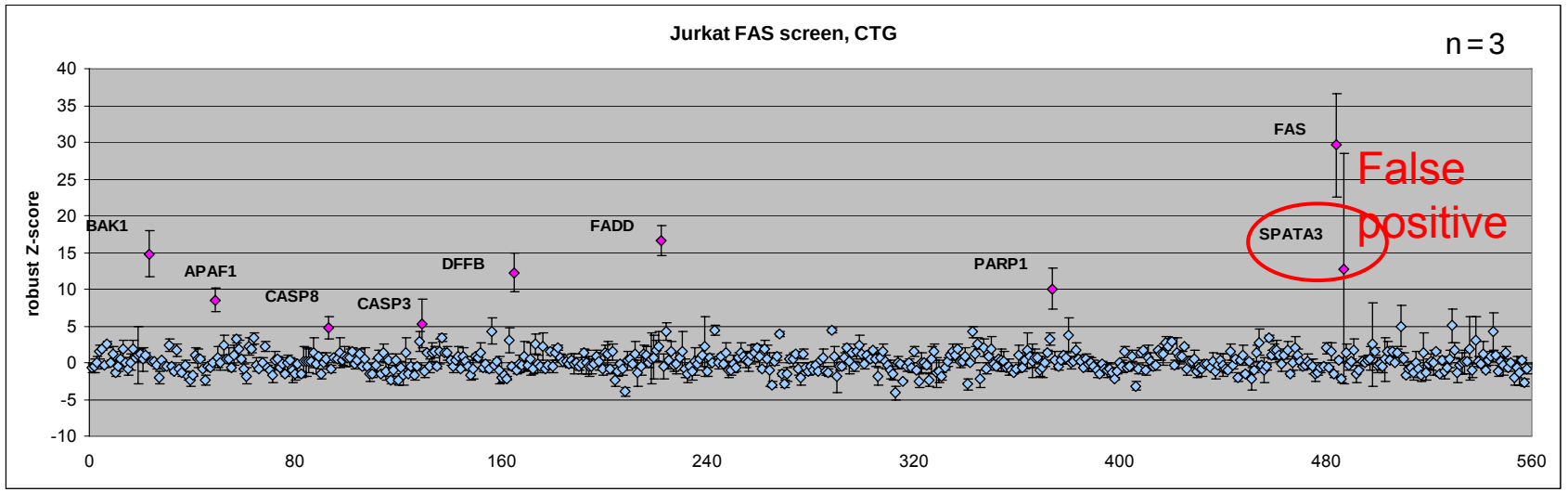
FAS-mediated apoptosis – assay setup



Optimization of assay parameters

- siRNA concentration
- siRNA type (siGENOME®, ON-TARGETplus®, Dharmacon)
- Cell number during Nucleofection®
- Cell plating density
- Culture volume
- Time point of apoptosis induction
- Inducer concentration
- Induction time before analysis
- Choice of assays (Cell titer, caspase activity)

Primary Screen Data, Cell Titer Glo Assay



Data generated in collaboration with DHARMACON

- Hits sorted by mean of rZ-Scores of three screens
- Very good correlation between the three screens
- One apparent false positive (identified by screen repetition)

siRNA FAS-Apoptosis Screen in Jurkat T Lymphocytes

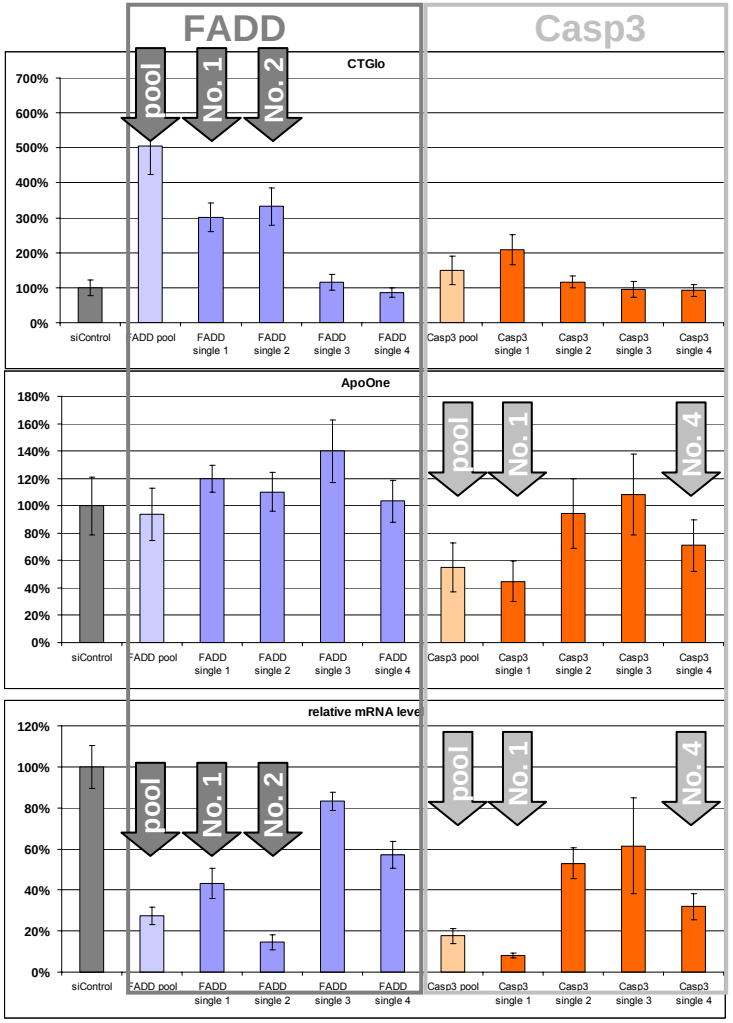
Deconvolution of pools, phenotype vs. knockdown

FADD

- Strong mRNA KD helps protect from cell death (see cell titer)

Casp3

- Strong mRNA KD reduces caspase activation (ApoOne)



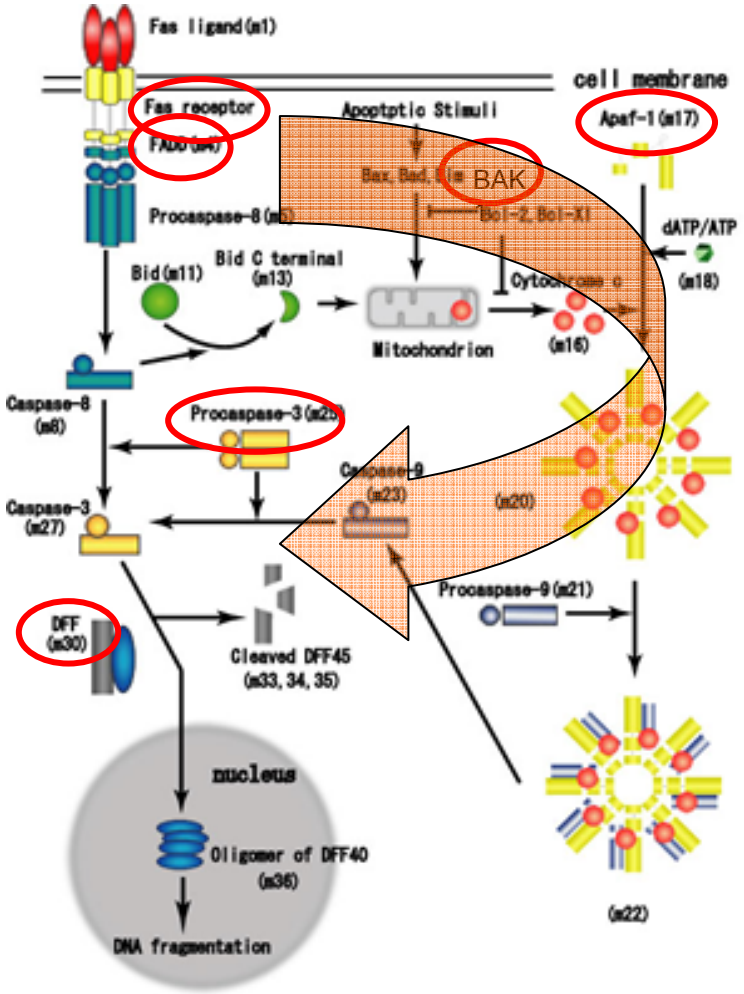
CellTiter-Glo®, Promega corp.

Apo-One® Promega corp.

mRNA

Data generated in collaboration with DHARMACON

siRNA FAS-Apoptosis Screen in Jurkat T Lymphocytes



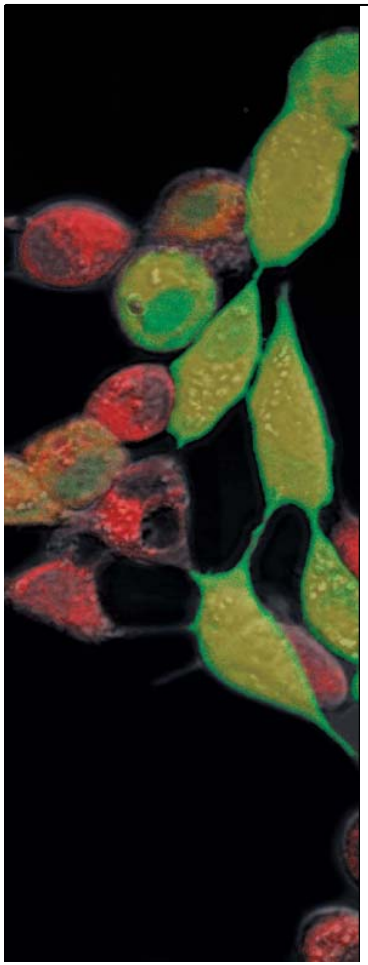
Cell titer
FAS
FADD
BAK1
DFFB
PARP1
APAF1
CASP3
TNFRSF1A
TRIM35
CASP8

ApoOne/ cell titer
BAK1
FAS
CASP3
APAF1
FADD
ALOX12
ITGB2
LY86
LTBR
TP53INP1

Hit list indicates that FAS apoptosis in Jurkat might primarily use the mitochondrial route

Data generated in collaboration with DHARMACON

Cell Engineering in High Throughput



Agenda

- High-Throughput Applications Based on Cell Engineering
- Functional siRNA Screening
  - Kinome/Cell Cycle siRNA Screening in Human Umbilical Vein Endothelial Cells
  - Functional Fas-apoptosis Screen in Jurkat T Lymphocytes
- **Cell Engineering with Non-Nucleic Acid Molecules**

The Rationale Behind Non-nucleic Acid Delivery

- Nucleofector® Technology - Accepted leadership for nucleic acid delivery in primary cells and hard-to-transfect cell lines
- Efficient delivery of other molecules extends the range of addressable research applications – including **high throughput**
- Current study covers
  - Small organic molecules
  - Peptides
  - Recombinant proteins and antibodies

Applications

- Small organic molecules:
  - Extension / prioritization of lead candidate pipeline by pharma customers by in-vitro validation of compounds transfected by Nucleofection®
  - Allow customers to screen / validate compounds not permeating membranes
  - Extending available pool of compounds beyond Ro5 (Lipinski)
- Peptides
  - Delivery of peptides for target and pathway validation
  - Enable systems biology approaches by complementing RNAi analysis

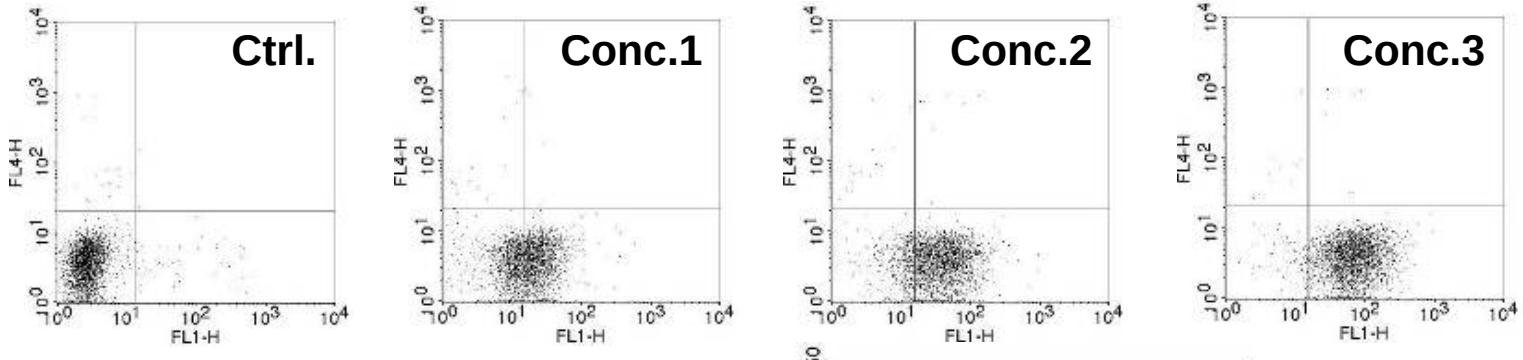
Lonza

Applications

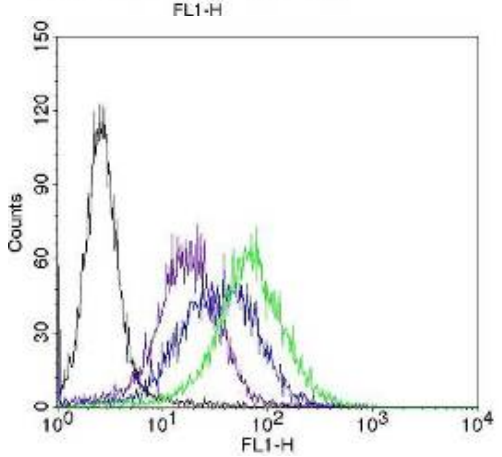
- Proteins
  - Use as a tool for functional validation of pathways
  - Functional in-vitro validation of recombinant proteins
- Antibodies
  - Use as specific intracellular labels or functional entities (e.g. inhibitory)
  - Intracellular trafficking & pathway validation

Small Organic Molecule Nucleofection®

Organic molecules per se not crossing the cell membrane are homogenously delivered by Nucleofection® in a concentration dependent way



Flow cytometry analysis of K562 cells transfected with carboxyfluorescein



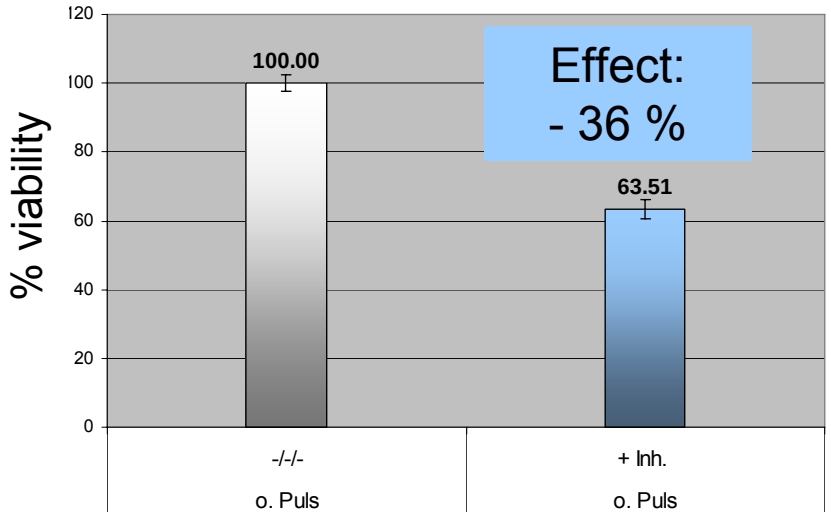
Ctrl.
Conc.1
Conc.2
Conc.3

Small Organic Molecule Nucleofection®

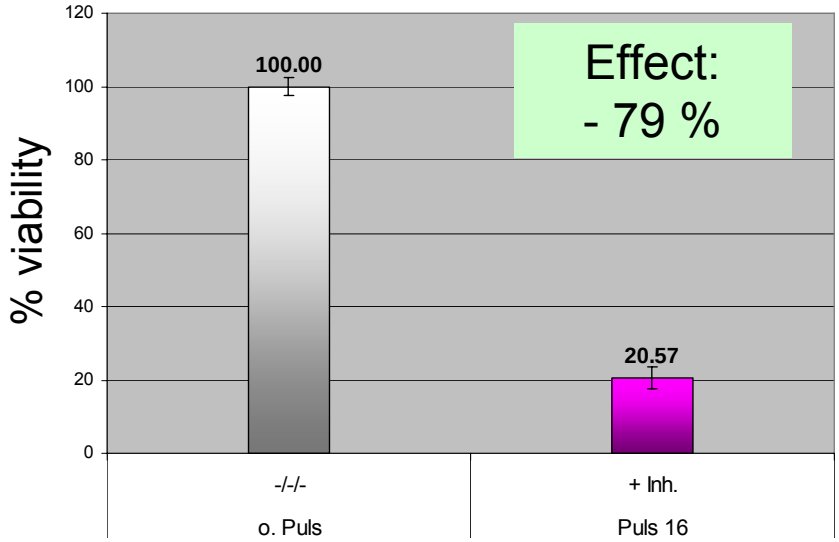
Transfected organic molecules exhibit functional in-vitro activity

- Viability analysis of Jurkat cells transfected with staurosporin

Effect of extracellular compound

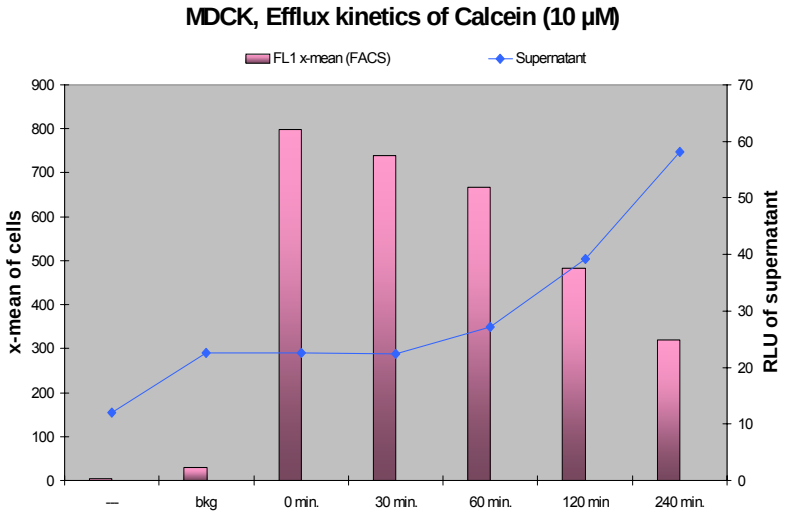


Effect of transfected compound

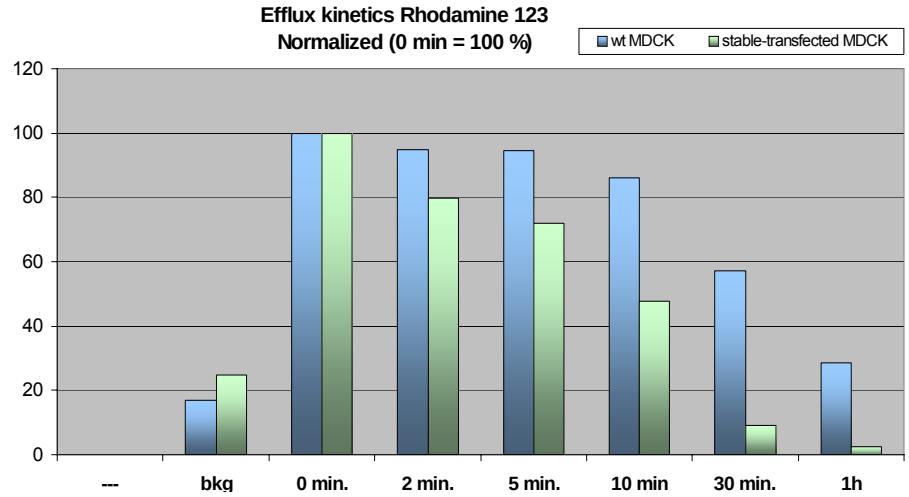


Small Organic Molecule Nucleofection®

Transfected organic molecules are specifically excreted via drug transporter proteins



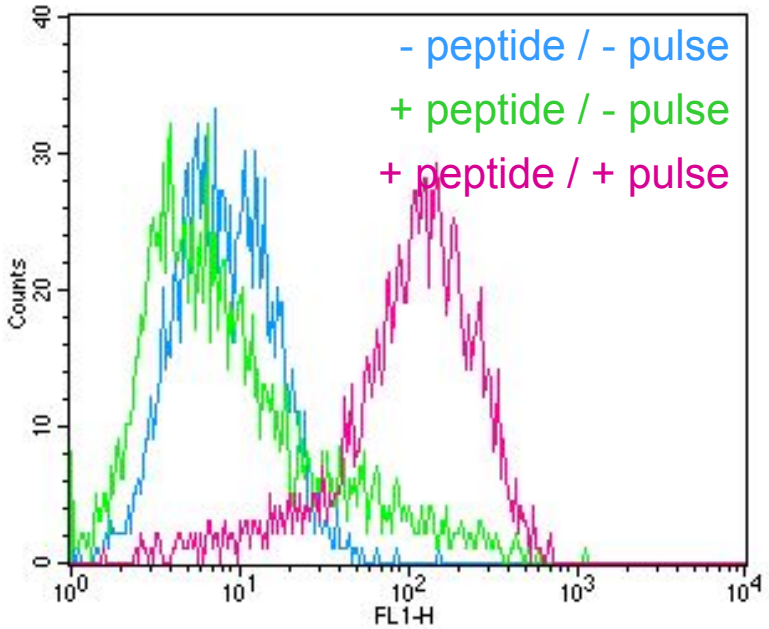
Time course fluorescence intensity analysis of wt MDCK after Nucleofection® with calcein



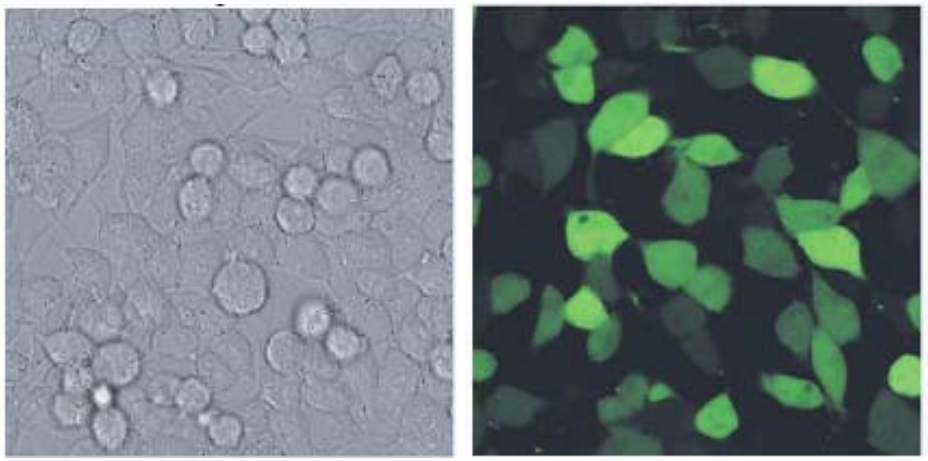
Efflux kinetic comparison of wt MDCK vs. MDCK over-expressing MDR1 transporter

Peptide Nucleofection®

Peptides from 8 to 36 amino acids are efficiently delivered by Nucleofection®



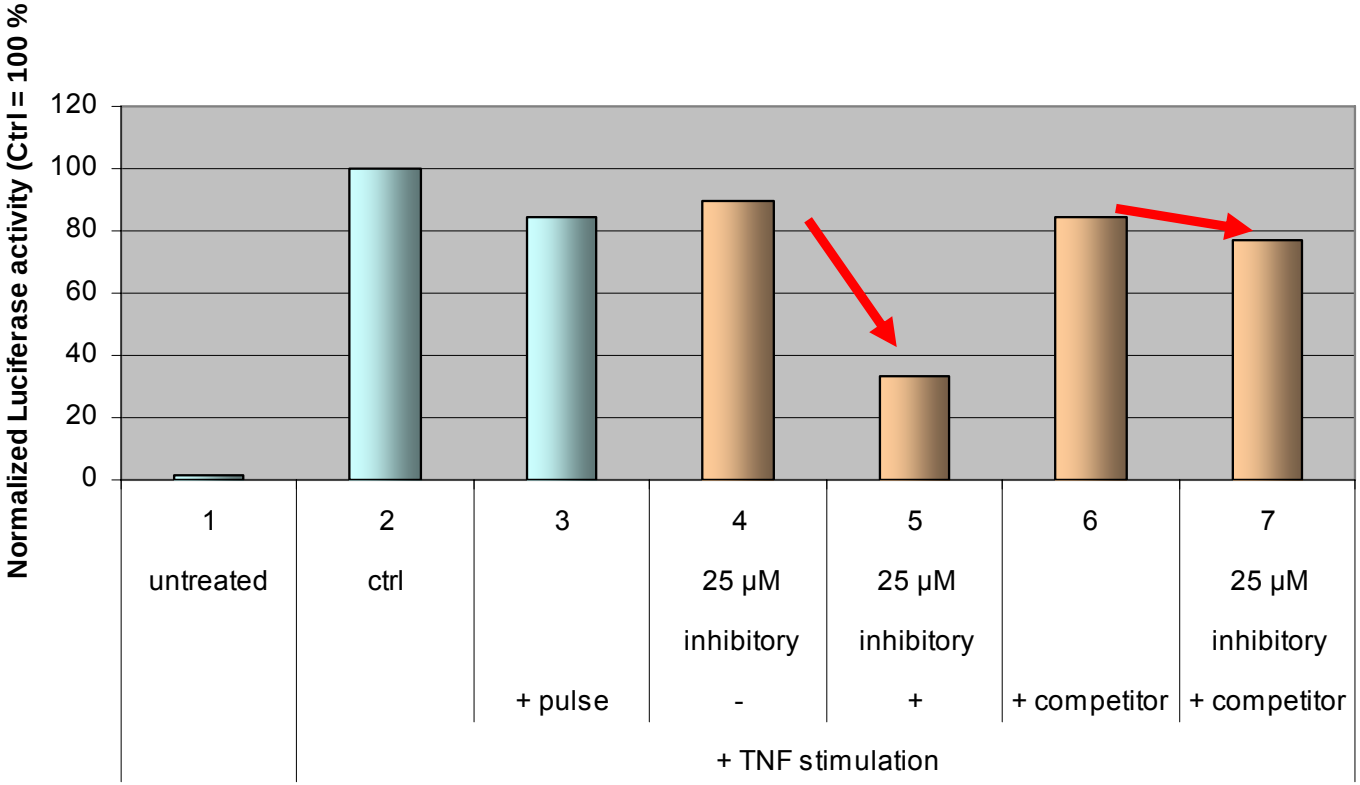
SH-SY5Y cells transfected with fluorescently tagged 8 AA-peptide (flow cytometry analysis)



SH-SY5Y cells 2 hours post Nucleofection® with fluorescently tagged 8 AA-peptide (confocal images)

Peptide Nucleofection®

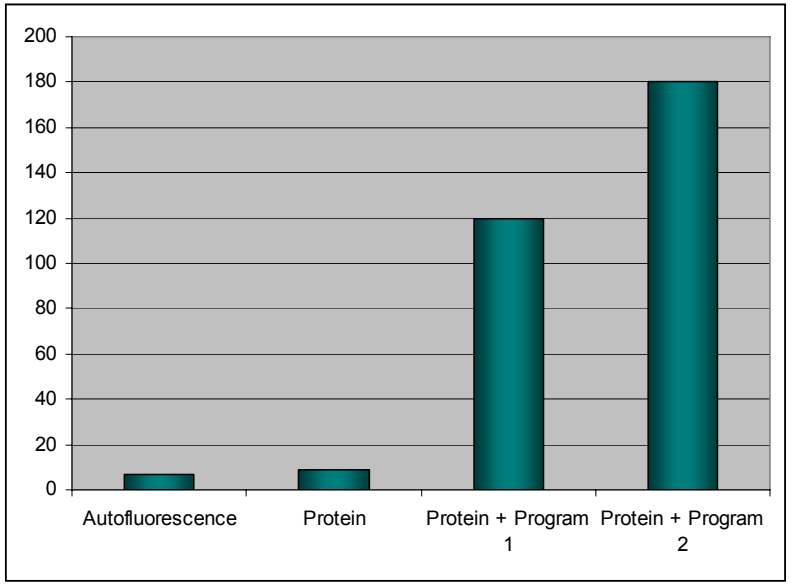
Transfected peptides exhibit functional activity



CHO-K1 Cells stably transfected with a Luciferase reporter of NF-κB pathway activity. Cells were stimulated using Tumor Necrosis Factor (TNF) and luciferase activity was assessed 30 minutes after peptide delivery.

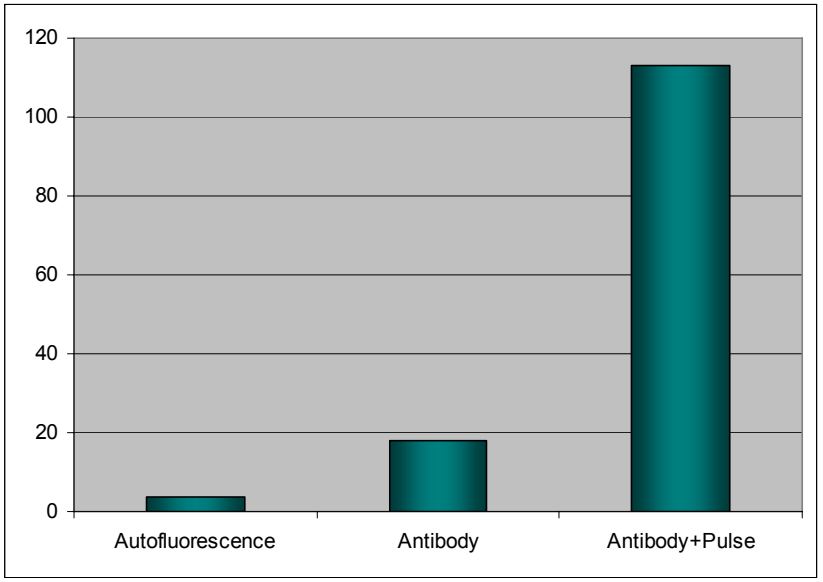
Protein Nucleofection®

Recombinant proteins are efficiently delivered by Nucleofection®



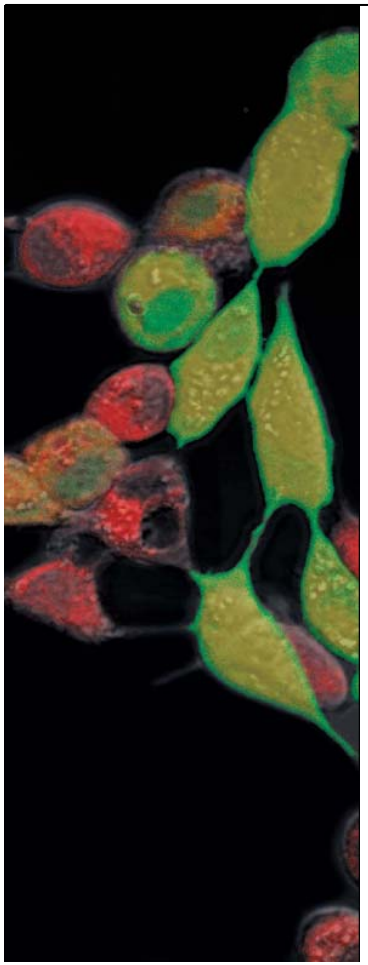
Jurkat cells transfected with fluorescently tagged ovalbumin

Full size antibodies are delivered by Nucleofection®



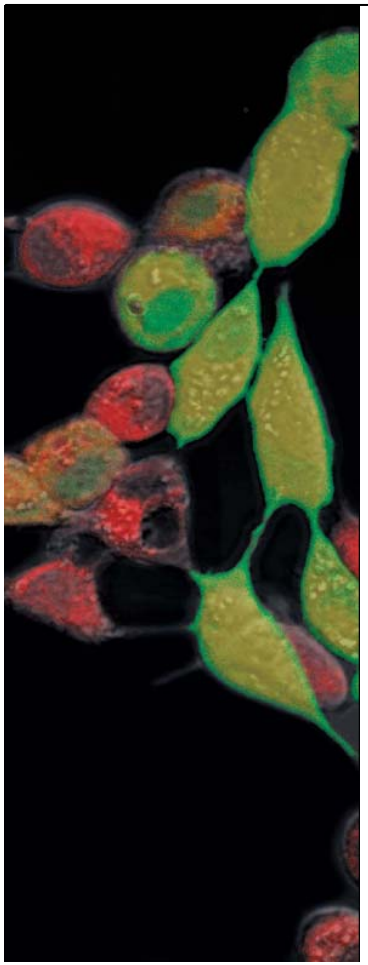
HeLa cells transfected with fluorescently tagged GαMIgG

Summary



- Manipulation of eukaryotic cells by introducing nucleic acids and other substrates goes HT
- Meaningful screening needs careful optimization and standardization of cell culture workflows and assay conditions
- Functional siRNA Nucleofection® screening campaigns in difficult-to-transfect cell types relevant for bio-medical research are feasible while maintaining cellular functions

Summary



- Reproducible and comfirmable hits were identified for:
  - Cell proliferation in the HUVEC cell model for angiogenesis
  - Fas-mediated apoptosis pathway in the T lymphocyte model Jurkat cells
- Upcoming applications for cellular delivery of non-nucleic acids

Acknowledgements

Thermo Fisher Scientific

Dharmacon products, Lafayette, CO

Devin Leake

Schering AG, Berlin

Claudia Merz

Amaxa, a Lonza company

Nicole Spottke
Sheila Offizier
Sandra Domzalski
Sonja Spicker
Markus Zumbansen
Ludger Altrogge
Qui Van
Daniela Litzenberger
Yvonne Strübing
Michael Kazinski

Tecan Symposium 2008

Thank you for your attention!

Herbert Müller-Hartmann
Director R&D
amaxa AG, a Lonza company